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(54) **Method of enhancing the regeneration of injured nerves and adhesive pharmaceutical formulation therefor.**

(57) The regeneration of injured nerves is enhanced by supplying collagenase to the zone of injury of the nerve. Growth of nerve sprouts over the zone of injury is aided by the presence of effective amounts of collagenase during the regeneration process. If the nerve has been severed, collagenase is supplied to the ends of the proximal and distal stumps. A nerve graft may be interposed between the stumps. Natural fibrin has been used as glue to join nerve stumps, and collagenase is effective when used in admixture with fibrin.

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Considerable success has been achieved in recent years in the repair of injured nerves, including those wherein complete severance of a nerve trunk has occurred. Microsurgery has enhanced the ability to match nerve ends precisely, but itself introduces additional trauma. Success of nerve repair is uncertain at best, not only because of surgical difficulties but also because of the many interrelated biological events occurring at the site of injury and beyond the site of injury.

Functional recovery after nerve repair is dependent on structural and functional events in the peripheral as well as the central nervous system. The pattern of cellular and biochemical events required to achieve useful sensory and motor regeneration after a nerve injury is complex. Among necessary factors are survival of the nerve cell body, initiation of the sprouting process, growth of sprouts over the zone of injury, reinnervation of endoneurial tubes in the distal segment, reinnervation of peripheral targets and maturation of nerve fibers/target structures.

Some of these problems can be approached surgically. The surgeon can influence the result by the way he or she handles the damaged nerve and by the method chosen for reconstruction. Other factors susceptible to influence are the timing of surgery and postoperative reeducation and rehabilitation. At present the remaining components of the regeneration process are more or less out of reach, but some may prove to be critical factors to address in the future.

The present invention deals with problems surrounding growth of nerve sprouts over the zone of injury, whether the injury results in total severance of a nerve trunk, or the condition known as "neuroma in continuity" where damage is caused by crushing; bruising, or partial laceration of the nerve.

In accordance with this invention, regeneration of injured nerves is enhanced by supplying an effective amount of collagenase to the zone of injury of the nerve during the regeneration process. In one embodiment, collagenase is mixed with fibrin or a fibrin precursor, which is then used as a glue between severed nerve ends. In another embodiment, a pharmaceutical kit is made up containing fibrin or a fibrin precursor and collagenase, either premixed, or separate for mixing at time of surgery. The invention is useful when the stubs of severed nerves are to be reunited either directly or by interposition of a nerve graft.

## Clinical Aspects of Nerve Repair

In order to facilitate understanding of the intricacies of nerve surgery and the factors that affect nerve regeneration, the following discussion is provided.

### Terminology

There are certain accepted surgical anatomical terminologies covering basic patterns of intraneural structures of importance for the surgical procedure. Nerve trunks have been defined as monofascicular (cross-section consists of one large fascicle), oligofascicular (crosssection consists of a few fascicles) and polyfascicular (the nerve consists of many smaller fascicles) (Millesi & Terzis, 1984). A fascicle is a small bundle of nerve fibers. The perineurium is the connective-tissue sheath that surrounds a fascicle. The epineurium is the external connective-tissue sheath of a nerve trunk. Connective-tissue contains collagen as a major component.

### Role of Collagen

Although several types of collagen form part of the peripheral nerve (Pleasure, 1984; Thomas & olsson, 1984), their role in the functional process of nerve conduction is unknown. Collagen occurs at two locations in peripheral nerve, the endoneurium and the perineurium. The collagen at each site is different (Thomas, 1963; Thomas & Olsson, 1984). It is known that the collagen content of peripheral nerve increases after complete transsection of the nerve (Holmes & Young, 1942; Millesi, 1977; Sunderland, 1968). Furthermore, the deposition of collagen in the gap between the cut ends of the nerve prevents axonal sprouting, regrowth and rejoining (Millesi, 1977; Pleasure et al., 1974). Millesi (1977) suggests that scar formation proceeds from the epineurium. He therefore recommends stripping the epineurium to reduce this process. However, recent studies (Eather et al., 1986) show that transsection of the ischiadic nerve in rats leads to increases in collagen concentration 2.5 mm proximal and distal to the injury. This indicates that the region around the transsection is involved in the process of collagen production. Thus, the production of collagen may have negative effects, but also serves essential functions in the rebuilding and regeneration of injured nerves.

The consequences of introducing collagenase into the biololically active zone of regeneration cannot be judged a priori. It has been suggested (Pinnelli U.S. Patents 4,524,065 and 4,645,668) that mammalian scars (which contain large amounts of collagen) be prevented by administering collagenase directly into the affected area during the healing process, or be dissolved by administering directly into the lesion. Pinnell states that purified collagenase has been demonstrated to be relatively safe even in large doses in contact with human blood vessels, nerves and bones (presumably Pinnell refers to intact nerves), but there is no suggestion that it be used in the repair of injured nerves or that it would enhance nerve regeneration. It may be postulated that in the present

invention one function of collagenase when supplied to the site of a nerve trauma may be to break down collagen as it is formed or after it has formed, but it is not apparent that this is the actual mechanism, or whether a favorable effect should outweigh an unfavorable effect, or whether collagenase is providing or contributing to other functions that result in overall enhancement of nerve regeneration. As stated earlier, the pattern of cellular and biochemical events required to achieve useful sensory and motor regeneration after a nerve injury is complex.

Timing

Discussions of primary versus secondary nerve repair are often based upon the time course of metabolic changes in the nerve cell body following nerve injury: after nerve transsection the nerve cell body is believed to present an optimal metabolic potential 2-3 weeks following the injury (Ducker, 1980; Ducker et al., 1969) and we know that a "conditioning lesion" preceding a second lesion might help to increase the outgrowth rate of axons considerably. In addition, structural and functional changes in the distal nerve segment following transsection may create optimal conditions for regeneration a couple of weeks following the initial trauma. These facts, together with clinical experience of nerve injuries seen in the Second World War (for reviews see omer & Spinner, 1980), may form a theoretical base for recommending delayed primary repair. However, one has to keep in mind that nerve injuries seen in war are quite different from the civilian injuries usually seen in our hospitals. War injuries usually involve considerable soft tissue trauma which makes it impossible to carry out nerve surgery before the surrounding tissues have healed, the inflammatory reaction has declined and demarcation of the ultimate level of nerve injury is clear. The nature of the wound rather than aspects of nerve cell body metabolism might have been the true reason for recommending secondary nerve repair in such instances (Ducker, 1981).

Crushing and laceration of nerve trunks are sometimes seen in civilian accidents too. In these cases, the nerve should be repaired secondarily when the demarcation of the nerve injury is obvious and adequate levels for scars/neuroma excision can be defined. However, in sharp, cleancut nerve injuries there are good reasons to recommend primary nerve repair. In the initial stage an exact orientation of the cut nerve ends is usually possible to achieve by the help of local landmarks like epineural vessels and a well-preserved fascicular pattern in the cut face of the nerve. At this stage the ends can still be sutured without tension. At a later stage, on the other hand, both the nerve segments have retracted and a great deal of scar tissue usually has to be resected (cut out). Tension at the suture line is difficult or impossible to avoid, and often a nerve graft has to be interposed.

Thus, considering the total situation, the ideal nerve suture is carried out as a primary procedure, a suggestion which is supported by experimental and clinical data (Grabb, 1968; Grabb et al., 1970; Muller & Grubel, 1981). With increasing preoperative delay the results could be expected to become progressively worse, and particularly liable to be affected by shrinkage and fibrosis of the distal nerve segment as well as degeneration of end organs.

Following nerve transsection the corresponding muscles atrophy rapidly and after 2 years muscle fibers may fragment and disintegrate. If reinnervation of the muscle occurs after one year, function can at best be poor, while a delay of 18-24 months causes irreversible changes in the muscle cells with no hope for return of motor function at the time of regeneration (Wilgis, 1982). Sensory organs seem to be more resistant to denervation than muscles and the final extent of sensory recovery has been reported to have little correlation with the time of injury to nerve repair (Onne, 1962).

Techniques for Nerve Repair

The choice of technique for nerve repair has been the subject of a great deal of debate in the literature. With the introduction of microsurgical techniques, it became possible to dissect, identify, map and even suture in a selective way, individual fascicular components of nerve trunk. Since axonal misdirection at the suture line is a principal problem, there was great hope that microsurgical techniques would improve the results. However, the tissue trauma associated with resection of the epineurium and extensive interneural dissections might per se induce microhemorrhages, oedema and fibrosis, interfering with axonal growth. The mechanical advantage of exact matching of fascicular structures has therefore to be balanced against possible harmful effects of the microsurgical trauma as such.

The purpose of all nerve repair techniques is to restore continuity of the nerve trunk, including all its elements, in order to achieve optimal reinnervation of the end organs. According to Millesi and Terzis (1984). the four basic steps of nerve repair can be defined as:

1. Preparation of the stumps, often involving resection or interfascicular dissection with separation of individual fascicles or groups of fascicles.
2. Approximation, with special reference to the length of the gap between the stumps as well as the amount of tension present.
3. Co-aptation of the nerve stumps. Co-aptation describes the opposition of corresponding nerve ends with special attention to bringing the cross-section of the fascicles into optimal contact. A

direct co-aptation (neurorrhaphy) can oppose stump to stump, fascicle to fascicle, or fascicle group to fascicle group in the corresponding ends. An indirect co-aptation can be performed by interposing a nerve graft.

4. Maintenance of co-aptation, involving the use of, for example, stitchest glue or a natural fibrin clot as glue.

Epineural Repair

Co-aptation of the nerve stumps by suturing the external epineurium is a classic method of nerve repair (Zachary & Holmes, 1946; Zachary, 1954; Edshage, 1964; Moberg, 1964; Braun, 1980; Snyder, 1981; Wilgis, 1982). An important step is the initial debridement of the nerve edges, which can be carried out by the use of soft membranous material wrapped circumferentially around the nerve to make the end firm enough to be cut with a scalpel or a pair of scissors. Cooling of the end has been used clinically (Edshage & Niebauer, 1966) and experimentally (de Medinaceli et al., 1983) to ensure sharp resection surfaces and facilitate the co-aptation. If the nerve has been sharply cut by the damage (glass, knife), there is usually no reason for further debridement before the repair is performed. The cut surface of the nerve may show protrusion of fascicular contents; if not too extensive, this should be accepted in order to avoid further trauma. Landmarks such as longitudinal epineural blood vessels are identified to ensure a correct rotation of the nerve stumps, and the fascicural pattern of the cut ends should be identified under high magnification, to further ensure correct matching of the ends when the suture is performed. The sutures are placed circumferentially in the epineurium of both stumps, initially at points where external landmarks make the correct rotation crystal clear. Further stitches are then placed around the circumference to secure and maintain the initial orientation. Due to postoperative edema, the nerve ends swell considerably during the first few days, and if the sutures are oo tight the ends will be strangulated. It is therefore important to make the sutures very loose. The number of sutures should be as few as possible, and no more than are needed to hold the ends close enough together with sufficient strength.

The advantage of the epineural suture technique is its simplicity and the minimal dissection trauma required. However, the technique does not ensure an absolutely correct matching of the fascicular structures over the nerve trunk. It was demonstrated by Edshage (1964) that the epineural suture technique may cause misalignment and considerable displacement of fascicles in spite of a perfect superficial appearance of the epineural adaptation.

Fascicular Repair

The object of fascicular repair, or more correctly ligroup fascicular repairl" is to achieve an optimal orientation by approximating and adapting fascicles or groups of fascicles individually (Sunderland, 1981; Kurze, 1964; Smith, 1964; Bora, 1967; Hakstian, 1968; Grabb et al., 1970; Millesi, 1973; Cabaud et al., 1976, 1980; Ito et al., 1976; van Beek & Kleinert, 1977; Terzis & Strauch, 1978; Lilla et al., 1979; Terzis, 1979; Tupper, 1980; Kline et al., 1981; Kutz et al., 1981). Fascicular groups are carefully freed by dissection under high magnificationj and the epineural tissue is resected over a short distance from the cut nerve.

Corresponding fascicular structures in both cut nerve ends should be inspected under high magnification, and co-aptation with exact matching of the fascicular groups is accomplished by placing 9-0 or 10-0 sutures in the interfascicular epineurium. Co-aptation by placing suture material in the perineurial sheath of individual fascicles is associated with extensive dissection trauma and makes sense only in nerves with few fascicles. The risk of damaging fascicles should be realized. Sutures penetrating the perineurium might induce microherniation of endoneurial contents and may delay restoration of an optimal endoneurial environment.

With the introduction of microsurgical techniques, the fascicular repair technique became popular, and vast clinical experience has now been gained. The repair does not resist much tension, and can therefore usually be carried out only as a primary procedure when no resection is reqiured. Its advantage is the possibility of achieving an optimal matching of corresponding fascicular components. Resection of epineural tissue serves to remove the most reactive connective tissue of the nerve and can facilitate the fascicular matching. However, resection of epineurium combined with separation of fascicular groups may induce considerable tissue trauma; including vascular injury and postoperative edema. The method has therefore the potential disadvantage of surgical trauma added to the original injury.

Fascicular repair requires optical magnification and can be carried out only by a skilled and experienced microsurgeon.

Nerve Grafting

Direct suture of the ends of a severed or lacerated nerve is not always possible to perform. When a nerve transsection is treated secondarily, it is normally necessary to resect a scarred area around the site of a lesion in order to achieve fresh resection surfaces. After this is done, the nerve ends cannot always be brought together without considerable tension. Advanced lesions, including damage to a segment of a

nerve, may result in a gap in the continuity of the nerve trunk.

Although tension can to some extent be overcome by mobilization of the nerve ends and flexion of adjacent joints, it has become apparent over recent years that tension at a suture line is disadvantageous for axonal growth. Even a slight tension can interfere with intraneural microvascular flow, compromising the nutrition of cellular components in both nerve ends. It has also been demonstrated that tension at the suture line increases scar tissue formation and decreases the quality of axonal regeneration (Millest et al., 1972a; 1976; Samii & Wallenberg, 1972; Orgel & Terzis, 1977; Miyamoto & Tsuge, 1981a; b; Millest & Meissl, 1981). Tension reduces the transsectional area of the fascicles, thereby increasing normal endoneurial fluid pressure. on the other hand, minimal tension is not necessarily disadvantageous to axonal growth since such directed mechanical "microforces" might help to create longitudinal polarization of the fibrin clot occurring between two cut nerve ends, thus providing contact guidance for the advancing sprouts. In chamber experiments where a gap is left between the nerve ends, contractile forces in the fibrin clot contribute to the creation of a longitudinally-oriented stroma guiding axons growing toward the distal nerve segment.

Since experimental and clinical experience show that too much tension at the suture line is disadvantageous for axonal regrowth, most authors today prefer to avoid tension by bridging the gap with nerve grafts. Although this procedure has created new opportunities to achieve functionally good results even in severe nerve injuries (Millest, 1977, 1980y 1984; Millest et al., 1972b, 1976; Wilgis, 1982), not all authors agree on the critical length of the defect which should indicate the use of a nerve graft. At a panel discussion on this subject (Millesif 1977). the opinions varied from 1.5 to 2 cm (Brunelli, Freilinger, Samil, Buck-Gramcko) to 4 mm (Kutz & Wilgis) and 6-7 cm (Urbaniak & Gaul).

Regeneration through nerve grafts has been studied experimentally in rabbits (Hudson et al., 1972) and rats (Miyamoto et al., 1981; Lundborg et al., 1982; MacKinnon, 1986). Extensive compartmentation has been observed at both the proximal and distal anastamoses (Hudson et al., 1972) and along the body of the graft (MacKinnon, 1986). Extra-fascicular fibers have been observed growing in the epineurium of the graft along its whole length (4 cm in rats) (MacKinnon, 1986). Although fiber counts suggested that these fibers never made functional connections. By 4 to 6 months postoperatively, the total number of fibers in the proximal segment had become constant, while there was still an increased number of smaller diameter fibers in the graft and distal segments. More fibers were present in the graft than in the distal segment indicating axonal branching at the first suture line and actual loss of fibers at the second suture line. No correlation was found between length of graft (rat peroneal nerve - length up to 2.5 cm) and number/maturation of regenerating fibers (Miyamoto et al., 1981).

Survival of Graft

The purpose of introducing grafts between the two ends of a cut nerve is to offer mechanical guidelines as well as an optimal environment for the advancing sprouts. In this respects the Schwann cells of the grafts and their basal laminae play an essential role. Laminin, located in the basal lamina of Schwann cells, is known to promote neurite growth and there are reasons to believe that certain proteins synthesized by the Schwann cells exert a neuronotrophic effect. If a thin nerve graft is placed in a healthy well-vascularized bed, it will survive and will be able to fulfill this purpose. It has been demonstrated by isotope techniques that most transplanted Schwann cells in such a situation survive, multiply, form Bungner bands and remain confined to the grafted segment (Aguayo et al., 1976a, b, 1979; Charron et al., 1976; Aguayo & Bray, 1980; Aguayor 1981). During the first day the graft survives by diffusion from the surrounding tissues. It is then revascularized rapidly, starting on the third postoperative day (Almgren, 1974). Thicker grafts have difficulties in surviving because of longer diffusion distances and delayed revascularization. The so-called "trunk graft" used in the past (for historical review, see Wilgis, 1982) usually showed a central necrosis because of its thickness.

Interfascicular Nerve Grafts

Millesi and his colleagues have shown that a gap in continuity in a nerve trunk is best treated with interfascicular nerve graftsy performed with the aid of microsurgical techniques (Millest et al., 1972b, 1976). The technical details of this procedure have been described in many excellent reviews (Millesi et al., 1972a, 1976; Millesi, 1977v 1980, 1981a, b. 1984; Wilgis, 1982). It is usually performed as a secondary procedure at a time when both the retracted nerve ends may be united by abandoned scar formations. Briefly, the dissection procedure is performed from normal to abnormal tissues. The epineurium is incised to make possible the identification of groups of fascicles. Separate groups are dissected free and traced towards the site of injury. At the point where the fascicles lose their normal appearance and run into the neuroma, the group is transsected. The epineurium is excised over a distance of 1-1.5 cm from the resection borders. In order to avoid a circumferential scar; each fascicular group should be transsected at a different level.

The transsectional surfaces are studied under

high magnification, and the patterns are mapped in order to identify corresponding fascicular groups. This process may be associated with considerable problems since the fascicular pattern of a nerve changes continuously along the course of the nerve. Moreover, the fasicular pattern of the graft does not correspond to the fascicular pattern of the nerve ends.

In nerves with fascicles arranged in groups, corresponding fascicle groups should be united by individual nerve grafts (interfascicular nerve grafts). In polyfascicular nerves without group arrangement, the fascicles may be aistributed diffusely over the crosssectional area, an arrangement which is particularly common proximally at the root level or the brachial plexus. In such cases, each sector of the cross-section should be covered by a nerve graft until the whole cross-section is complete, socalled sectoral nerve grafting (Millesi, 1980).

Source of Nerve Graft

The most common choice is the sural nerve, which has an appropriate thickness and which can be harvested in considerable lengths from both lower limbs. The sural nerve has a varying pattern ranging from monofascicular to poly-fascicular, and only a few branches (Millesi, 1981b). other suitable choices are the lateral or medial antebrachial cutaneous nerves (McFarlane & Myers, 1976). The terminal parts of the posterior interosseous nerves have been used as a graft in terminal lesion of digital nerves (Wilgis & Maxwell, 1979). In rarer instances, the superficial radial or lateral femoral cutaneous nerves can be used. The graft should be reserved to avoid loss of axons through branchings (Ansselin & Davey, 1986).

According to the concept of grafting, no tension at all should be tolerated at the suture lines between the graft and host nerves. The aptation could therefore be maintained by only one or two stitches of very tiny suture material (e.g., 10-0 nylon) and even fibrin clotting may be sufficient to maintain the co-aptation if tension is completely avoided (Millesi, 1980; Futami et al., 1983; Kuderna, 1985).

A problem can sometimes occur at the distal suture line where scar formation may present an obstacle to the advance of the axonal sprouts.

Free Vascularized Nerve Grafts

It is known from experimental studies that single segmental extrinsic vessels approaching a nerve trunk can maintain the intrinsic microcirculation in the nerve over long distances. It is tempting to apply this to microvascular techniques and insert free vascularized nerve grafts in gaps in nerve continuity: if the recipient bed is heavily scarred, a conventional non-vascularized nerve graft may not be optimally vascularized. In experiments on rats, the number and average diameter of regenerating axons has been found to be greater in vascularized nerve grafts than in free non-vascularized grafts (Koshima & Harii, 1981). and regenerating axons have been reported to grow at considerably greater speed in vascularized nerve grafts than in free nerve grafts (Koshima et al., 1981).

The concept of vascularized nerve grafts was introduced by Taylor and Ham (1976) and the technique has more recently been described by, among others, Breidenbach and Terzis (1984, 1987), Boney et al. (1984). and Gilbert (1984). Five cases of segmental vascularized nerve grafts bridging scarred beds for digital sensory nerve reconstruction where previous non-vascularized nerve grafts had failed were reported by Rose and Kowalski (1985). They reported good recovery of sensibility, including average static two-point discrimination of around 9 mm.

Because of the expense, time and technical expertise required, vascularized nerve grafts must be reserved for very special occasions, primarily cases where normal revascularization of the grafts cannot be expected to take place. Among other possible advantages of vascularized nerve grafts used in a scarred recipient bed might be their ability to act as vascular carriers of non-vascularized nerve graft (Breidenbach & Terzis, 1984).

Nerve Lesion in Continuity

Peripheral nerve lesions with preserved continuity of the nerve trunk but loss of distal function to varying extents constitute one of the greatest challenges in peripheral nerve surgery. Such partial loss of function might result from subtotal nerve transsectionst blunt nerve trauma or traction injuries. Various fiber components of the nerve trunk can, in such casest present all stages from simple neurapraxia (Sunderland grade 1) to neurotmesis (Sunderland grades 3-5). While some axons may be transsected or ruptured, others may be compressed by interneural scar or compromised by vascular insufficiency. The approach to this type of injury, also called "neuroma in continuity,," is extremely difficult. In these cases the surgeon may supply collagenase to the zone of injury, in accordance with the present invention. Surgical exploration, including neurolysis or resection and reconstruction, might also be indicated if the permanent situation cannot be accepted. In such cases, applying collagenase at the point of surgical intervention facilitates nerve regeneration.

The surgeon, if experienced with the type of lesion, may by inspection under high magnification be able to gauge to some extent which fascicles are healthy and should be spared and which are damaged and should be resected and replaced. However, with this method the findings can often be misleading and methods for intraoperative assessment of fiber func-

tion with electrophysiological recording techniques have been developed. Kline et al. (1968, 19691 1972) introduced techniques for intraoperative neurophysiological assessment of the extent of the lesion by stimulating and recording from whole nerves. With the development of microsurgical techniques, more refined methods for stimulation and recording from individual fascicles or fascicular groups became available. Hakstian (1968) introduced a method of stimulating motor and sensory fascicles separately in the proximal and distal nerve segments to improve accuracy in experimental nerve suture, and similar techniques have long been utilized to assess the quality of nerve regeneration following various types of nerve repair (Terzis et al., 1975, 1976; Terzis & Williams, 1976).

On the basis of these investigations, single fascicular recordings have been successfully used as an intraoperative diagnostic tool in microsurgical repair of nerve lesions in continuity (Kline & Nulsen, 1972; Williams & Terzis, 1976; Kline, 1980; Terzis et al., 1980). According to these principles, single fascicles or, if that is not possible, groups of fascicles are freed by dissection and isolated proximal and distal to the lesion. Each individual fascicle is lifted onto stimulating and recording electrodes, electrical stimuli are delivered proximally and a nerve compound action potential (CAP) is recorded distally to the lesion. On the basis of the conduction velocity as well as the shape and amplitude of the wave form, the degree of nerve injury can be assessed and a decision made regarding the treatment of the fascicle. If there is a measurable response, intraneural neurolysis might be justified while absence of any response might indicate resection and grafting of the damaged fascicle.

Hentz et al. (1966) introduced a new principle for intraoperative recording of the small magnetic field induced by the passage of a compound action potential along a nerve. He demonstrated experimentally that virtually all parameters of the magnetic signals recorded correlated closely with the clinically useful parameters of the standard recorded CAP, and that the recording could be made without removing the nerve from its normal physiological environment, since suspension on electrodes in air is not required.

Treatment of nerve lesions in continuity must be determined in the first place by clinical examination as well as inspection and palpation of the damaged part of the nerve in situ. However, combinations of whole nerve and single fascicular recordings might, if adequate equipment and expertise are available, contribute to a correct ultimate judgment of the lesion.

The accompanying drawings show results of tests of the effect of collagenase in the regeneration of severed nerves in groups of test animals (rats)1 as compared with controls.

Figure I shows motor performance.

Figure II shows amplitudes of evoked muscle potential.

Figure III shows for a single rat representative traces of evoked spinal potential and of evoked muscle potential.

Figure IV shows velocities of motor nerve conduction.

Figure V presents photomicrographs of nerve cross-sections.

Figure VI shows total number of myelinized nerve fibers.

Collagenase (Clostridiopeptidase A) is an enzyme produced from the bacterium clostridium hystolyticum. Highly purified collagenase uniquely cleaves bonds in the collagen structure permitting other enzymes to act on the resulting molecular fragments.

Purified collagenase, used in the following worky is free of detectable caseinase and nonspecific protease activity. It can be obtained from Advance Biofactures Corporation, Lynbrook, New York, sold under the trademark "Nucleolysin." The frozen enzyme is thawed and diluted with normal saline solution plus 2 mM calcium chloride to the desired concentration. Enzyme activityl given in ABC units, is determined using an insoluble substrate, undenatured bovine tendon, according to a modification of the method of Mandl et al. (Arch. Biochem. Biophys. 74:465-475, 1958). A unit of activity corresponds to the release of ninhydrin reactive material equivalent to nanomoles leucine equivalents released in one minute from undenatured collagen. The collagenase is obtained from culture of a special strain of clostridium histolyticum and purified by a chromatographic technique.

The following information demonstrates and exemplifies various aspects of the invention.

Materials and Methods

The right sciatic nerve of the rat was used as an experimental model in two series (I = collagenase in the silicone model; II = collagenase in epidural suture with fibrin). A total of 42 rats (male, weight about 300 g) were examined in these series.

The operation was performed at time = 0 days under general anesthesia (40-50 mg/kg body weight). The sciatic nerve was visualized through a lateral incision on the right side. Transsection of the nerve was effected with a clean cut by microscissors, prior to which 3 marking sutures (9-0. Ethilon) were put in place. The latter were inserted before transsection as means of orientation, to obtain like conditions of rotation in all animals. No immobilization was carried out postoperatively. Preliminary tests established that the anesthetic used produces no alteration in properties of nerve conduction in the test region. In addition, it was demonstrated that collagenase is not inhibited by either fibrin glue or clot material, and collagenase

caused no alteration in nerve conduction properties in the test region.

Effect of Collagenase on Result of Nerve Regeneration in the Silicone Model (Series I)

In the first part of the study (Series I) the hypothesis was tested in the silicone tube model. The experimental group (Series IB) consisted of 12 animals and the control group (Series IA) likewise of 12 animals.

The two nerve ends were inserted into a silicone tube 5 mm in length (outside diameter 2 mm; inside diameter 1.5 mm - Wehabo, Dusseldorf) which had previously been filled in the center with an average of 1.7 mg collagen (type I bovine collagen - Sigma No. C-9879) in a length of I mm, until they struck the collagen mass. The nerve was anchored and secured to the silicone tube by 3 holding sutures (9-0), proximally as well as distally. This was followed by the injection of 0.05 ml collagenase (50 units) through the silicone tube into the center of the collagen or saline 0.05 ml in the control group.

Complete clinical and neurophysiological examinations were performed preoperatively, on the 7th, 12th, 15th, 19th, 25th, 32nd, 40th, 60th, (80th) and 90th postoperative days in all animals of the two groups. The hind paws were recorded photographically at the same time intervals, to rule out autonomous denervation reactions.

At the end of the observation period, 6 treated nerves each of the experimental group (IB) and of the control group (IA) were processed further for histology and morphometry.

Effect of Collagenase in Epidural Suture Technique Combined with Fibrin Glue (Series II)

Approach and transsection of the nerve were carried out in the same way as in Series I. The epidural suture was made with 6 x 9-0 nonresorbable sutures (Ethilon™).

Following suture, the suture region was thoroughly coated with a fibrin/collagenase mixture (1 ml of this mixture consisted of: 1000 I.U. aprotinin; 50 I.U. thrombin; 1000 U. collagenase; total mixture applied 0.05 ml 50 U. collagenase). After the region of transsection was packed with this mixture, an additional injection of 0.01 ml (= 25 U. collagenase) was made into each of the proximal and distal nerve segments in a region of about 1 mm.

The 12 animals of the experimental group were treated identically to the 6 animals of the control group (fibrin glue without collagenase + saline).

Six treated nerves of the experimental group (IIB) and 3 of the control group (IIA) were examined histologically and morphometrically by the same methods as in Series I.

The exmaination intervals during the 90-day observation period were identical to those of Series I.

Clinical Findings

Fig. I shows the average motor performance of the treated right hind paw in Series I+II evaluated by deMedinaceli's method (SFI = sciatic functional index) 90 days after transsection of the right sciatic nerve. - 100% corresponds to complete paresis of the hind paw.

Motor performance after 90 days is significantly higher in the experimental group of Series II + I.

In Series I, after 90 days, 11 animals (total n 12) of the experimental group exhibited a normal toe spreading reflex, while in the control group this was detectable in only 5 animals (total n - 12).

In Series II, the toe-spreading reflex was observed on the right side in 10 animals (total n = 12) of the experimental group and in 3 animals of the control group (total n = 6).

Neurophysiologic Findings

Evoked Spinal Potential (SSEP Li)

The SSEP response, in particular the level of amplitude, was regarded as a measure of functioning afferent axons.

At test times t = 90 days, the amplitudes of evoked spinal responses showed higher amplitudes in the experimental groups of both series [IIA/B m: 7.33 uV/10.64 uV; s.d.: 3.83/1.63; $p < 0.162$) (IA/B m: 6.40B uV/11.058 uV; s.d.: 4.977/2.81; $p < 0.01$)]. Even at time t = 60 days, there were differences between experimental and control group in both series, although these were on the order of $p < 0.024$ (Series I) and $p < 0.023$ (Series II) in the t test.

On the 25th postoperative day, an evoked spinal potential was observed in 9 animals (total n = 12) of the experimental group IB (IIB: 10 animals; total n = 12), while an evoked spinal potential was detectable in 5 animals (total n = 12) in the control group of Series I and in 1 animal (total n = 6) in Series II.

CMAP (Amplitude of Evoked Muscle Potential)

Fig. II shows the results of the measurement of amplitudes at times t = 0 days, t = 60 days and t = 90 days in both series.

Level of amplitudes was measured in the intrinsic muscles of the foot (in mV) after stimulation of the sciatic nerve proximal to the site of transsection of the nerve. Compared with the respective control group, a highly significant increase in amplitude of the total muscle potential is striking in both collagenase-treated series [Group IA/B preop. m: 6.067 mV/5.792 mV; s.d.: 0.794/0.406; 16 degrees of freedom; $p < 0.338$.

90 days m: 0.227 mV/1.408 mV; s.d.: 0.403/0.571; 21 degrees of freedom; p < 0.0001) (Group II/ A/B preop. m: 4.58 mV/4.56 mV; s.d.: 1.3/0.55; 16 degrees of freedom; p < 0.954. 60 days m: 0.13 mv/1.07 mV; s.d.: 0.3/0.63; 16 degrees of freedom; p < 0.004. 90 days m: 1.52 mV/3.37 mV; s.d.: 0.44/0.75; 16 degrees of freedom; p < 0.0001)]. Means with standard deviation correspond to leads of a total of 42 animals. The highest amplitude values (3.37 mV) at the end of the observation period were obtained by the nerves which were treated with collagenase/suture + fibrin (Group IIB). These nerves exhibited values close to those prior to the operation (4.56mV).

SSEP and CMAP of One Animal

Fig. III shows representative potentials in rat No. 42, which was treated with collagenase.

The tracings are of representative leads of evoked spinal potential (SSEP L1. tracing on left) and of evoked muscle activity in the intrinsic muscles of the foot (CMAP, tracing on right) after stimulation of the sciatic nerve proximally (CMAP) and distally (SSEP) of the site of transsection after injection of collagenase in simultaneous epidural suture and fibrin coating. Immediately after transsection of the nerve, this animal (No. 42) developed a complete paresis of the foot, which showed clear clinical improvement from the 60th day. The filter setting was LF: 10 Hz + HF: 10 kHz, the SSEP (tracing on the left) corresponds to 64 averaged responses, the CMAP (tracing on the right) corresponds to a single stimulation of the nerve. Supramaximal stimuli were employed. If a threshold value determination was not possible because of complete paresis, we stimulated with 40 V. Note the distinct increase in amplitude in the CMAP on the 60th and 90th post-operative days, which was accompanied by clinical improvement. The clinical and neurophysiological test values of the unoperated opposite side were unremarkable.

Velocity of Motor Nerve Conduction (VNC)

In Series I (silicone model) the VNC was determined preoperatively in all animals. After 90 days a normal muscle potential following stimulation at two different locations of the right sciatic nerve was obtained in all animals of the collagenase group (n = 12). while in the control group this was possible in only 4 animals (n = 12).

After 60 days this was possible in 10 nerves of the experimental group and in no nerve of the control group.

In Series II (epidural suture with fibrin), the VNC was likewise determined in all animals, if possible.

After 90 days determination of the VNC was possible in all animals of the experimental group (IIB) and in all animals of the control group. However, the experimental group exhibited a distinctly higher VNC (IIB m: 27.42 m/s. s.d.: 4.62; IIA m: 19 m/s. s.d.: 5.29; 16 degrees of freedom; p < 0.003).

After 60 days determination of the VNC in the operated nerve was possible in all animals of the experimental group and in one animal of the control group (total n = 6).

There was no significant difference in preoperative values in the two groups (p < 0.593).

Thus, compared with all other groups of Series I + II, Group IIB also obtained the highest values in velocity of nerve conduction.

Fig. IV summarizes the means with standard deviation of the velocity of nerve conduction (m/sec) of Groups I + II after 90 days. The animals in which determination of the VNC was not possible because of total loss of potential were not included in the statistical analysis VNC. The two columns on the left show means with standard deviation in Group I. in which the effect of a total of 50 units of collagenase on regeneration was studied in the silicone model (control/collagenase m: 19.75/24.25 m/s; s.d.: 4.856/6.510; 14 degrees of freedom; p < 0.229). The two columns on the right show means with standard deviation in Group II, in which the effect of a total of 100 units of collagenase was studied in epidural nerve suture with fibrin glue (controls/collagenase m: 19/27.42 m/s; s.d.: 5.29/4.62; 16 degrees of freedom; p < 0.003). The distinct difference in the velocity of conduction of motor fibers of Group IIB, compared with Group IIA, is striking.

Morphometric Findings

Fig. V shows nerve cross-sections in various magnifications of a nerve which was treated with collagenase in the silicone model (A and B), and of an untreated normal nerve (C and D). (A=30x + B=1000x) Nerve cross-section 5 mm distal to nerve transsection after 3 months' regeneration. In the silicone model 50 units of collagenase were injected at time t = 0. (C + D) Nerve cross-sections of an untreated normal nerve; note the pronounced homogeneous myelinzation.

Fig. VI shows the total number of myelinized fibers in Series I and II in experimental and control groups. The count was at 5 mm distal to the original site of nerve transsection. The bars represent means with standard deviation.

With the use of collagenase in combination with fibrin and suture (Series II), the average count was on the order of 10,476 myelinized nerve fibers (s.d.: 1107); in the control group, it was on the order of 9,685 myelinized fibers (s.d.: 620). In the collagenase group of Series II there was an increased total fiber count at a low level of significance (p < 0.235).

With the same investigatory technique, a total count of 12,760 fibers (s.d.: 1553) was obtained in the

silicone model study, while a total fiber count of 9588 (s.d.: 2728) was found in the control group. The increase in the fiber count was significantly greater ($p < 0.047$) in the collagenase group of Series I.

Morphometry in Series I (silicone model) and II (suture + fibrin model), in terms of relative total fiber diameter, and percentage cluster of nerve fibers, in diameter groups ranging from 1.0 to 10 ~m in steps of 0.5 ~m, revealed no appreciable differences between collagenase-treated and control groups.

Concentration and Dosages

The invention is useful in the repair of injured nerves of humans and animals. Generally speaking, similar concentrations of collagenase can be used.

Concentrations of about 500 to 1,000 ABC units collagenase per ml are ordinarily satisfactory, although lower or higher concentrations; for example down to 200 u/ml or less and up to 2,500 u/ml or more, may be employed. The collagenase will be used in a pharmaceutically acceptable medium, such as normal saline solution, which may be enhanced with any chosen adjuvant, such as CaCl2 in a concentration of 2 mM.

When a mixture of collagenase with fibrin is to be used, the concentration of collagenase will generally be in the same ranges, e.g. about 200 to about 2,500 ABC units per ml, preferably about 500 to 1,500 ABC units per ml. The concentration of fibrin (or fibrin precursors) will be sufficient to form a somewhat viscous liquid solution or suspension of fibrin adequate to act as adhesive for the nerve ends, and will ordinarily be within the range of about 0.05 to about 0.5 gram fibrin per ml.

Since fibrin is acting as a glue, the ratio of collagenase to fibrin is not critical. Ratios of collagenase (in ABC units) to fibrin (in grams) may range from about 500 or less to about 50,000 or more.

Fibrin can be supplied as such or preferably by way of fibrin precursors, as by use of an admixture of a fibrinogen with sufficient thrombin to convert it to fibrin. Choice of ratios of fibrinogen to thrombin to cause clotting by fibrin formation are within the skill of the art. One can use, for example, aprotinin and thrombin in a ratio of about 20 I.U. aprotinin to one I.U. thrombin, in a concentration per ml of 1,000 I.U. of the former and 50 I.U. of the latter. Whole blood, or blood fractions containing fibrinogens, can be used with thrombin as fibrin precursors. The patient's own blood can be admixed with an amount of thrombin that will cause clotting in situ and thus gluing.

The dosage, i.e. quantity of collagenase, applied to the affected area will be dependent on the need. For a simple severed peripheral nerve trunk easily sutured, 50 to 100 units will ordinarily be sufficient. The physician will use his judgment in the amount of collagenase used for more extensive repairs.

Pharmaceutical Kit

For convenient use during surgery when fibrin is to be used as adhesive, collagenase and fibrin can be packaged together and sold as a pharmaceutical kit. The kit can contain a mixture of the two components, with collagenase present in a concentration preferably ranging from about 500 to 1,500 units/ml. One suitable formulation is: 1 ml containing 1,000 I.U. aprotinin; 50 I.U. thrombin, 1,000 ABC units collagenase. For greater shelf life, and to provide the surgeon with freedom to choose amounts and concentrations, collagenase and fibrin or fibrin precursors can be kept separate in the kit, to be mixed at the time of surgery. It is desirable for fibrin formation in the presence of collagenase to occur at the nerve site, which is readily accomplished by use of a kit containing quantities of collagenase, fibrinogen, and thrombin kept separate from each other, to be mixed immediately before use at the site of nerve repair.

In view of the preceding description, further modifications and alternative embodiments of the instant invention will be apparent to those skilled in the art. Accordingly, the preceding descriptions and examples are to be construed as explanatory and illustrative only and are for the purpose of teaching and enabling those skilled in the art to practice this invention. It should be understood that the amount of the collagenase required will vary. Suitable amounts in a given situation can be determined by the physician and the following factors, among others, should be considered: the nature of the nerve trauma being treated, the surgical methods used for repair, the concentration of collagenase in the solution or in the fibrin, the type of collagenase used, the nature of the tissue adjacent to the site of injury being repaired.

## Claims

1. A method of enhancing the regeneration of injured nerves which comprises supplying an effective amount of collagenase to the zone of injury of the nerve during the regeneration process.

2. A method according to Claim 1 wherein the nerve has been severed and collagenase is supplied to the ends of the proximal and distal stumps.

3. A method according to Claim 2 wherein fibrin containing collagenase is used as adhesive for the stumps.

4. A method according to Claim 3 wherein the ends are sutured.

5. A method according to Claim 4 wherein the sut-

ured region is coated with fibrin/collagenase mixture.

6. An adhesive formulation comprising fibrin as adhesive and collagenase present in an amount and concentration effective to enhance regeneration and rejoining of a severed nerve when said formulation is used as adhesive or fibrin precursor for the stumps.

7. An adhesive formulation according to Claim 6 wherein the fibrin or precursor therof is present in a concentration of 0.05 to 0.5g of fibrin per ml.

8. An adhesive formulation according to Claim 6 or Claim 7 wherein the collagenase is present in a concentration of 500 to 1,500 ABC units/ml.

9. A pharmaceutical kit for surgical use comprising fibrin adhesive or fibrin precursor and collagenase.

10. A kit according to Claim 9 wherein the fibrin or fibrin precursor and collagenase are admixed.

11. A kit according to Claim 10 wherein the collagenase is present in a concentration of 500 to 11500 ABC units/ml.

12. A kit according to Claim 10 or Claim 11 wherein the fibrin or precursor thereof is present in a concentration of 0.05 to 0.5g of fibrin per ml.

13. A kit according to Claim 8 wherein the fibrin or fibrin precursor and collagenase are separate.

14. A kit according to Claim 13 wherein the fibrin precursors comprise fibrinogen and thrombin.

15. A method according to Claim 1 wherein injury has resulted in neuroma in continuity.

16. A method according to Claim 1 wherein the stumps of individual severed fascicles or fascicle groups are separately co-apted.

17. A method according to Claim 2 wherein a nerve graft is interposed between the stumps.

18. A method according to Claim 17 wherein interfascicular nerve grafts are employed.

19. A method according to Claim 1 wherein collagenase is supplied in a pharmaceutically acceptable medium containing about 200 to 2,500 ABC units of collagenase/ml.

20. A method according to Claim 19 wherein the medium comprises normal saline.

21. A method according to Claim 1 wherein collagenase is supplied in a pharmaceutically acceptable medium containing 500 to 1,000 ABC units of collagenase/ml.

22. Use of collagenase for the manufacture of a medicament for enhancing the regeneration of injured nerves.

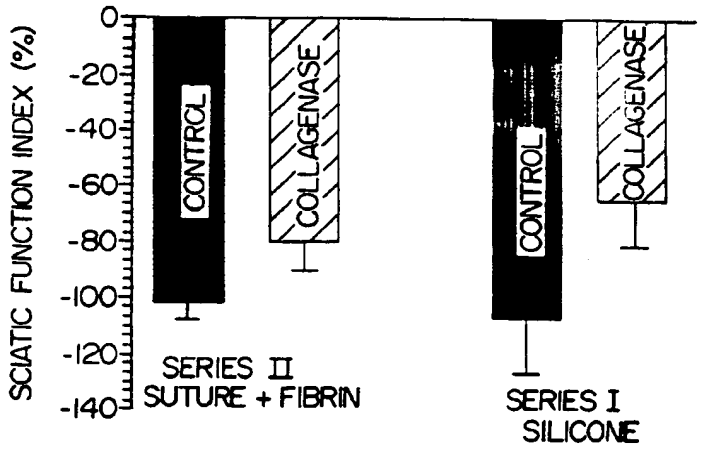
SCIATIC FUNCTION INDEX (%)
0
-20
-40
-60
-80
-100
-120
-140
CONTROL
COLLAGENASE
CONTROL
COLLAGENASE
SERIES II
SUTURE + FIBRIN
SERIES I
SILICONE

FIG. 1

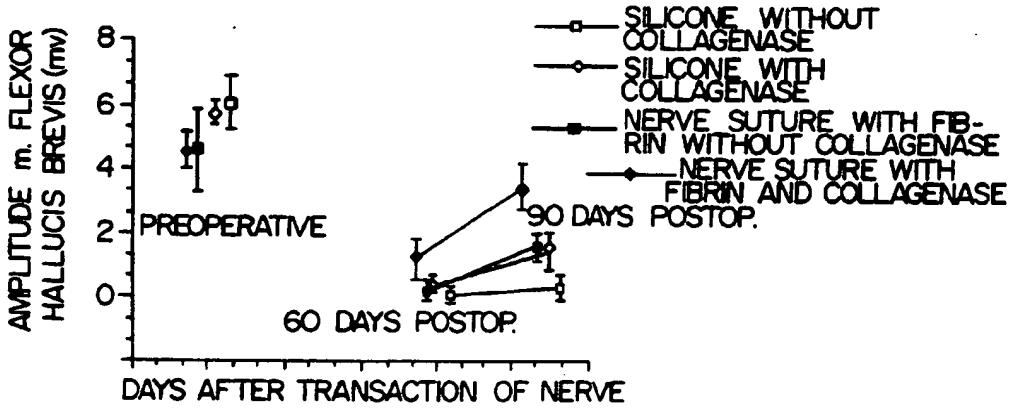
AMPLITUDE m. FLEXOR HALLUCIS BREVIS (mv)
8
6
4
2
0
PREOPERATIVE
60 DAYS POSTOP.
90 DAYS POSTOP.
DAYS AFTER TRANSACTION OF NERVE
SILICONE WITHOUT COLLAGENASE
SILICONE WITH COLLAGENASE
NERVE SUTURE WITH FIBRIN WITHOUT COLLAGENASE
NERVE SUTURE WITH FIBRIN AND COLLAGENASE

FIG. 2

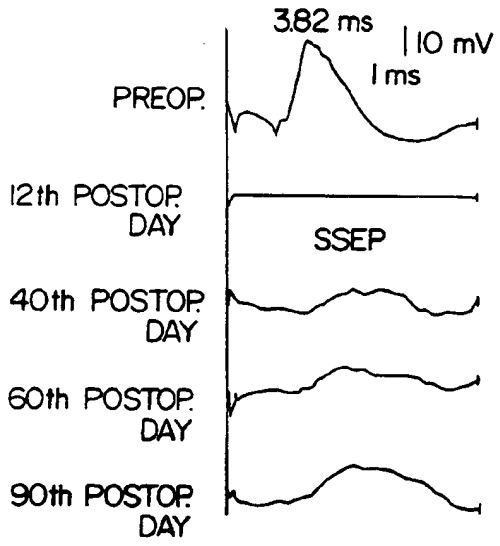


FIG. 3A

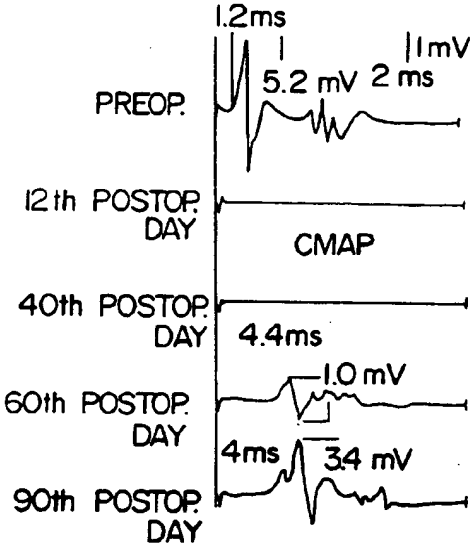


FIG. 3B

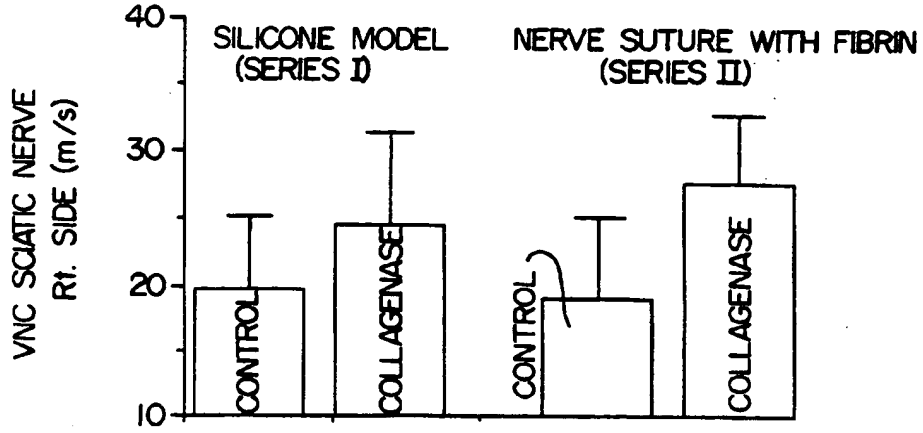


FIG. 4

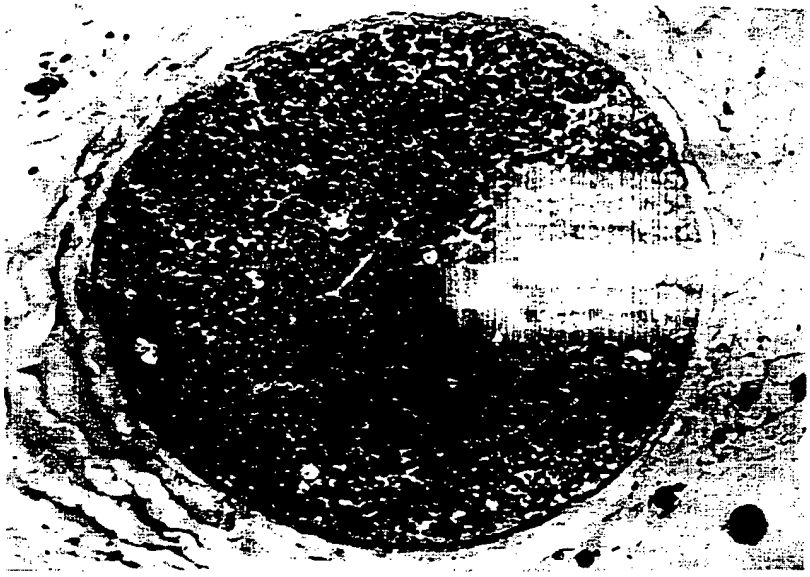

FIG. 5A

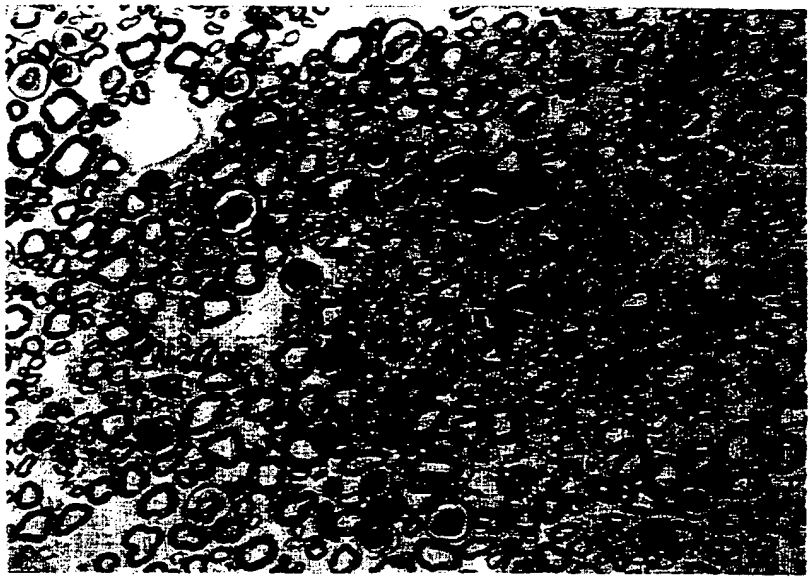

FIG. 5B

FIG. 5C

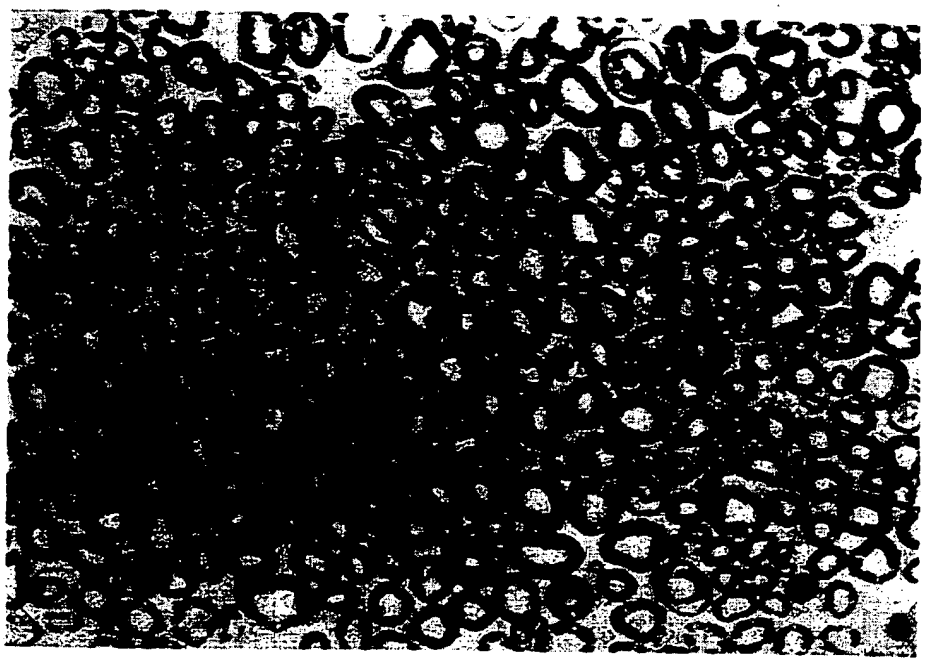

FIG. 5D

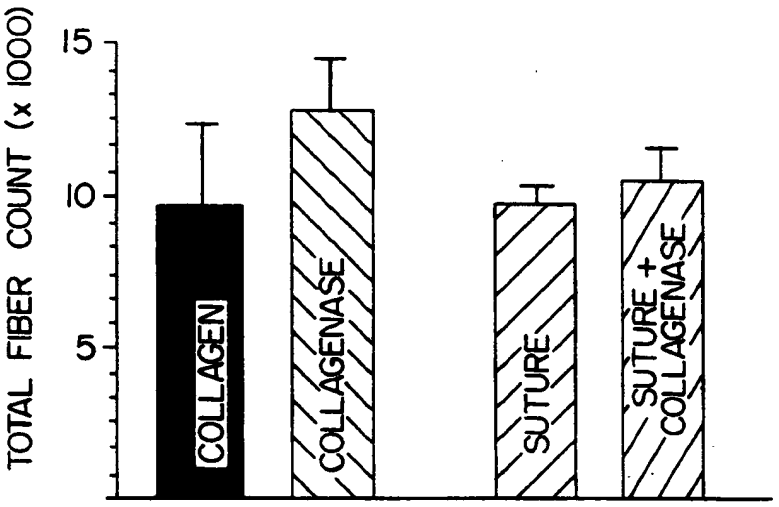
TOTAL FIBER COUNT (x 1000)
15
10
5
COLLAGEN
COLLAGENASE
SUTURE
SUTURE + COLLAGENASE

FIG. 6

European Patent Office

# PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number

EP 91309137.7

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.5) |
|---|---|---|---|
| A | GB - A - 1 251 398 (WORTHINGTON BIOCHEMICAL) * Claims; page 1, lines 11-43 * | 6,22 | A 61 K 37/54<br>A 61 K 37/48<br>A 61 L 25/00 |
| D,A | US - A - 4 524 065 (PINNELL) * Claims * | 6,8,22 | |
| D,A | US - A - 4 645 668 (PINNELL) * Claims * | 6 | |

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

A 61 K
A 61 L

## INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: 6-14,22
Claims searched incompletely: -
Claims not searched: 1-5,15-21
Reason for the limitation of the search:

Art. 52(4) EPC

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| VIENNA | 17-12-1991 | BECKER |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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B 01 J 31/00, C 12 N 11/04
B 01 D 13/04

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06.07.82 US 395768

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(54) **Fibrin gel containing catalytic active substances.**

(57) A fibrin gel containing therein means for retaining the shape against deformation and at least one catalytical active substance.



EP 0 187 894 A1

Croydon Printing Company Ltd.

0187894

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention relates to a filter with a definite pore size comprising fibrin and a process for the preparation thereof from fibrinogen. This invention also relates to a size selective process employing the filters of the invention.

## DISCUSSION OF PRIOR ART

Formation of fibrin gels by contacting fibrinogen with a coagulation enzyme has long been known. According to quotation no. 55888k in "Chemical Abstracts" Vol. 84, No. 9, 1.3.76, it is known to embed chloroplast ATPase ($CF_1$) in fibrin membrane, which is formed by fibrinogen-fibrin conversion in the presence of thrombin and is stabilized by blood-coagulation factor XIII under physiological conditions. This immobilized chloroplast ATPase has the potent ATPase activity, 0,3 µmoles phosphate/$mm^2$/min and does not cause the cold inactivation of the enzymic activity.

Furthermore quotation no. 126372h in Chem. Abstr. Vol. 88, No. 17, 24.4.78 reports of fibrin membranes being used for immobilization of enzymes (such as asparaginase) to stabilize the enzymes. Fibrinogen solutions are treated with thrombin at 30 to 40°C. Thin clot membranes are formed in the presence of calcium. The membranes are dried to a water content of less than 20%.

It has been observed that when a liquid is passed over the gel, permeation of the gel increasingly becomes difficult - sometimes to the point where permeation and passage of the liquid are rapidly diminished or cease. It was believed that the gel infrastructure was extremely

fragile and that the gel consisted of networks of channels and pores of varying size which were highly changeable, and highly dependent upon and variable with liquid or liquid mixtures passed thereover, especially one having solid particles.

It was therefore thought that such fibrin gel was not useful in separating components where the separation was effected solely on the basis of particle size.

Specifically, when investigating the fibrin formation from fibrinogen the interest was directed to the flow properties of fibrin gels. It has e.g. been shown before that the flow properties through silica gel as well as through agar and gelatin gels are such as for a viscous flow. It has also been shown earlier that the flow through a fibrin gel is dependent upon the ionic strength and fibrinogen concentration

in the preparation. In the investigations made, the permeability coefficient ($K_s$) of the fibrin gels was determined by Poiseuille's law as follows : (Darcy coefficient) $K_s = \frac{Q \cdot L \cdot n}{t \cdot A \cdot \Delta p}$ (1)

wherein Q is the flow through the gel in $cm^3$, A is the gel surface in $cm^2$, $\Delta p$ is the pressure difference in dynes/$cm^2$ (=0.1 $N/m^2$= 0.1 Pa), t is the time in seconds, L is the length of the gel in cm and n is the viscosity in poise (=0.1 Pa . s). Moreover, Kozeny-Carman has shown that the following relationship applies in a viscous or laminar flow in a capillary system:

$$m = \sqrt{\frac{K_o \cdot K_s}{\cos^2 \phi \cdot \varepsilon}} \qquad (2)$$

wherein m is the hydraulic radius ( $\frac{\text{wettable surface}}{\text{wettable circumference}}$ ). in cm, $K_o$ is a factor decided by the geometry of the capillaries, and $\phi$ is the orientation (angle) of the capillaries to the direction of flow, $\varepsilon$ is the partial share of liquid in the gel and r is the radius of the capillaries in cm. $\varepsilon$ can be calculated by means of the protein concentration and with a knowledge of the partial specific volume of the fibrinogen which is 0.72. For gels of the type concerned here $K_o$ and Cos $\phi$ cannot be calculated. In the theoretical calculations it has been assumed here that the capillaries are cylindrical and parallel to the direction of flow, which according to Madras et al brings the indicated formula to the following:

$$r = 2m = \sqrt{\frac{8 \cdot K_s}{\varepsilon}} \qquad (3)$$

The theoretical pore size is therefore 2r. By effective pore size we mean: the size at which particles of smaller size pass through the pores and particles of larger size are retained.

It has appeared from the tests that the clotting time (time of gel

formation) of the thrombin-fibrinogen mixture, here called Ct, is directly proportional to the flow (Q) through the gel. The flow (Q) has further been found to be inversely proportional to the fibrinogen concentration (C). Provided Q = 0, when $\frac{1}{C} = 0$ and Ct = 0, the equation (1) will have the following form:

$$K_s = \frac{k \cdot Ct \cdot K \cdot n}{C \cdot A \cdot P \cdot t} \qquad (4)$$

wherein k is a constant which is dependent on pH, ionic strength and calcium concentration and, moreover, is characteristic of the enzyme used in the gel formation, and Ct is the clotting time in seconds. The other symbols are the same as in equation (1). The term t is omitted when the flow is expressed in $cm^3/s$. According to this equation the permeability coefficient $K_s$ is thus directly proportional to the clotting time Ct and inversely proportional to the fibrinogen concentration.

By varying the pH between 6 and 10 the ionic strength between 0.05 and 0.5, the calcium ion concentration between 0 and 20 mM and/or the concentration of enzyme (e.g. thrombin, "Batroxobin" or "Arvin") between 0.01 and 10 NIH-units (or the corresponding units of other enzymes) per ml solution and the fibrinogen concentration from 0.1 and up to 40 g/l, preferably between 1 and 10 g/l, gels with $K_s$-values [calculated according to the equation (1)] between $10^{-7}$ and $10^{-12}$, preferably between $10^{-8}$ and $10^{-11}$, can be prepared. Calculated according to the equation (3), the corresponding average radii will be 0.03 - 9 μm, preferably 0.09 - 2.8 μm. If FXIII (a transamidation enzyme) and calcium ions are present in the gel formation the stability of the gels will be increased as covalent cross-

linkings will arise between the chains in the subunits of the gel matrix.

Thus, now it has been found according to the invention that these fibrin gels can be used as a filter. The filter according to the invention is characterized in that it is built of fibrin and the fibrin gel is in association with a shape-retaining means which retains the shape of at least one surface of said gel against deformation when contacted by a flowing liquid.

The filter of the invention has substantially uniform pores. By that is meant that the standard deviation of pore size is less than 15 percent, preferably less than 10 percent and in some instances less than 5 percent.

The pore size of the gel has, moreover, been found to be a function of the clotting parameters used in the gels' preparation, i.e., the pore size is varied by changing said parameters. The pore size is then proportional to the clotting time.

It has now been discovered, in accordance with the invention, that fibrin in gel form can be used as a filter if means are provided to retain the shape of at least one surface of the gel against deformation when the gel is contacted by a flowing medium such as a flowing liquid medium containing components to be separated. It has also been discovered, quite surprisingly, that the gel has substantially uniform pore sizes and that these pore sizes can be regulated simply by altering the process parameters employed for the formation of the gel

Specifically, it has been discovered, if the gel is in some way stabilized by a shape-retaining means, that the gel structure is preserved and the uniform pores therein function ideally as a filter medium.

Generally speaking, the gel is brought in contact with a shape-retaining means. The shape-retaining means can be a foraminous member such as a foraminous sheet member and is preferably disposed on or in association with an upper surface of the gel, preferably in contact with the gel either directly or through an adhesive or a graft. Since the foraminous member serves to preserve the shape and structure of the upper surface of the gel when the medium to be filtered contacts the same, the gel does not collapse, thereby allowing the uniform pores thereof to function ideally as a filter medium.

Foraminous members functioning as shape-retaining means can have virtually any size and shape, although they are preferably in the form of a sheet and preferably are substantially co-extensive with the upper surface of the gel. The foraminous sheet members can be in the form of a fibrous network such as in the form of a woven or non-woven or knitted fabric, the fibers of which can be natural or synthetic.

When the fibers of a foraminous sheet member are natural, they can be, for example, made of silk, wool, cotton, cellulose, hemp, jute or the like.

As synthetic fibers, there are contemplated in particular fibers made of nylon, polyester, polyolefin, fibers

made of vinyl polymers, acrylics such as polyacrylonitrile, rayon, to name a few.

The fibers generally have a thickness between 1 μm and 1000 μm, preferably between 10 and 20 μm, and are disposed in relationship to one another to define openings therebetween of between 0.01 and 5 mm, preferably between 0.05 and 1 mm, it being understood that the size of the openings between the fibers of the foraminous sheet is not especially critical, provided it allows passage therethrough of the medium to be filtered. It is preferred that as much fiber be in contact with or adhere to the gel as possible so as to insure maximum structural integrity of the surface of the gel initially to come in contact with the medium to be filtered.

Instead of using a fibrous foraminous member, one can use one made of wires, such as wires made of copper, tin, zinc, aluminum, glass, boron, titanium, steel, stainless steel, etc. The wires function analogously to the function performed by the fibers in providing structural integrity to at least one surface of the gel, preferably the upper surface or surface which is to be initially brought in contact with a mixture to be filtered. The interstices between the wires are of the same magnitude as the interstices between the fibers of a woven, non-woven or knitted fabric serving as a foraminous sheet member. The wires can be in the form of a screen, wire mesh or an expanded wire sheet and are preferably co-extensive with at least one side of the gel, preferably the upper surface.

The gel has uniform pores but owing to the manner by which the gel can be formed, can have uniform pores over a wide range. Preferably, the substantially uniform pores of the fibrin gel have a theoretical pore size or diameter in the range of about 0.003 to 9 μm, more preferably 0.009 to 2.8 μm.

The gel is formed by contacting fibrinogen with an enzyme, especially a coagulation enzyme. Particularly contemplated enzymes for use in forming a fibrin gel include thrombin, Batroxobin, Arvin, Eccarin, Staphylocoagulase, Papain, Trypsin, caterpillar venom enzyme, etc.

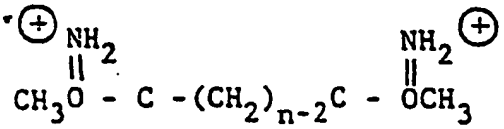
Generally speaking, the gel formation is effected at room temperature, although temperatures from -3°C up to 58°C can be employed. Preferably, the temperature is in the range of 0° to 40°C.

It is preferred that the gel be formed by contacting the fibrinogen with an enzyme in the presence of calcium ions. The calcium ion concentration can be up to 20 mM. The presence of calcium ions is not required in all instances. Where thrombin is employed as the coagulation enzyme, the gel can be formed in the absence of a calcium ion.

In forming the gel, there is generally employed 0.1 to $10^{-5}$ enzyme units per gram fibrinogen, preferably 10 to $10^{-3}$ enzyme units per unit weight fibrinogen. Following formation of the gel whose coagulation time is a function of the relative amount of enzyme to fibrinogen as well as the concentration of calcium ion, the gel is preferably hardened or

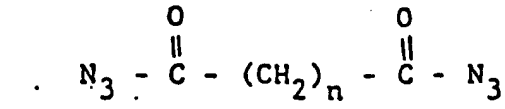
set by crosslinking the components thereof by contacting the gel with a crosslinking agent. Crosslinking agents contemplated include bis-imidates such as suberimidate, azides like tartryl di(ε-amino carproylazide), aryl dihalides like 4,4-difluoro-3, 3'-dinitrophenyl sulfone, glutardialdehyde, nitrenes, N,N'(4-azido-2-nitrophenyl)-cystamine dioxide, cupric di(1,10-phenanthroline), dithio bis-(succinimidyl propionate); N,N'-phenylene dimaleimide as well as polyethyleneimides and other bifunctional compounds, especially those known to crosslink with epsilon lysine, alpha amino groups, carboxy groups of aspartic and glutamic acids, and hydroxyl groups of amino acids in the protein chain (e.g. threonine and serine).

Bis-imidates which can be used include those of the formula



wherein n = 3 to 15 especially 3 to 10.

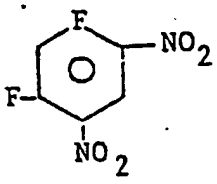
Azides which can be used include substituted and unsubstituted azides of the formula

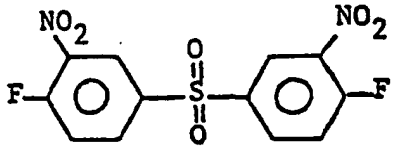


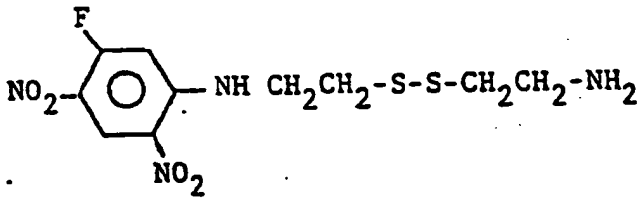
wherein n - 1 to 20 especially 1 to 15. Azides contemplated include those having a hetero atom in the chain, especially nitrogen. Also contemplated are hydroxy substituted azides.

Aryl dihalides which can be used include those having mono, poly and fused rings as well as rings joined by a

direct bond or through a methylene bridge or a sulfo bridge. The halogen of the halide can be fluorine, chlorine, or bromine. The compounds can be substituted by inert or functional groups such as nitro, or disulfide. Contemplated compounds include those where a functional group has replaced one of the halo substituents, e.g. nitro. Compounds contemplated include







Especially contemplated is glutardialdehyde.

Generally speaking, the crosslinking agent is employed in an amount of between 0.001% and 8 % by weight, preferably between 1 and 2 % by weight of the gel for 1-120 minutes. Crosslinking is effected at temperatures of between 10° and 40°C, preferably 20° to 25°C. After the hardened or crosslinked structure is obtained, the gel is usually washed free of extraneous material.

The gel in such hardened form is useful as a filter, i.e., without any foraminous sheet material. Preferably, however, the gel is formed on or in association with a shape-

retaining means such as a net, wire mesh or other sheet material and while in contact with such shape-retaining means is hardened by the use of a hardening or crosslinking agent.

Preferably, the gel is supported on its upper and lower surfaces by a shape-retaining means such as a foraminous sheet or the like, whereby to insure that the gel retains its shape during use as a filter.

This invention further contemplates a process for separating a first substance having a theoretical size of 0.003 to 9 μm from a second substance having a larger size which comprises passing a mixture of said first and second substances over a filter comprising fibrin in gel form and having pores of substantially uniform size, said filter having means for retaining the shape of at least one surface of said gel against deformation when contacted by a flowing medium, wherein the effective pore size of said fibrin gel is larger than the particle size of said first substance and smaller than the particle size of said second substance. Preferably, the pores of the gel have a theoretical size of 0.009 to 2.8 mm.

The filters of the invention are important, as they permit the separation of bacteria and viruses from mixtures containing the same. The ability to regulate the pore size and to achieve a gel of uniform pore size is an important and critical characteristic of the filters of the invention. These filters permit the separation of blood components, the separation of components of blood plasma, the removal of platelets from blood, the fractionation of cells and cell fragments and the separation of high molecular weight protein aggregates. In addition,

a variety of particles such as latex, silica, carbon and metallic particles may be separated over these filters. Components which can be separated include those shown in the table below:

TABLE A

| Material A Separated | Material from Which Material "A" is Separated | How Separated | | Effective Pore Size Range for Filter |
|---|---|---|---|---|
| | | Retained | Eluted | |
| Blood platelets | Blood plasma | X | | Below 1 µm |
| Red blood cells | " " | X | | 1 µm and below |
| Sendai virus | Culture medium | X | | 0.1 µm and below |
| " " | " " | | X | 0.2 µm and above |
| Liver mitochondria | Cyto plasma | X | | 0.5 µm and below |
| " " | " " | | X | 0.5 µm and above |
| Adeno virus | Culture medium | X | | 0.05 µm and below |
| " " | " " | | X | 0.1 µm and above |
| E. coli bacteria | " " | X | | 1 µm and below |
| FVIII complex | High molecular weight material (h.m.w) separated from low molecular weight material (l.m.w) | X (h.m.w) | X (l.m.w) | 0.05 µm and below |
| Blood leucocytes | Blood plasma | X | | 1 µm and below |
| Blood lymphocytes | " " | X | | 1 µm and below. |

Fibrin gel filters have above all the advantages over other gel filters that the pore size can be simply varied as desired. Moreover, the present filters have high flow rates at such pore sizes as can be used to remove very small particles, such as virus particles. In this respect, the filters of the invention are more suitable than known membrane filters and filters of polyacrylamide gels. The absence of absorption of protein on the filters is also an advantage as compared with certain other filters.

## DESCRIPTION OF SPECIFIC EMBODIMENTS

The process according to the invention for the preparation of gel filters is as described above, characterized in that a fibrinogen solution with preadjusting clotting parameters is mixed with a coagulation enzyme and the resulting mixture is made to clot in a form intended for the filter. It may be convenient to strengthen the fibrin gel formed during or after clotting by a shape-retaining means of greater strength than the gel which is preferably applied to the upper surface of the gel to be prepared and preferably to both the upper and lower surfaces thereof.

The shape-retaining means (reinforcing meshes) are preferably in the form of a net which is applied at the lower and preferably also at the upper surface in the mold in which the fibrinogen mixture is poured (cast). This mixture can preferably penetrate the net (foraminous sheet material) e.g. have its surface 10 µm-5mm, preferably 0.5-2 mm, from the net. The net can have a mesh width, for example, 10 µm to 5 mm, preferably 50 µm-1mm and the wire diameter can be, for example, 0.01-1.0 mm preferably 0.1-0.5 mm, where wires are employed as a shape-retaining means. In addition to the metallic wires noted

above, wires of natural fibers and plastics can also be employed. The filter can also be reinforced in other places than at the surfaces. It can, for example, be built on a foam material, such as a plastic foam, which can support part of the entire filter.

The filters of the invention, especially in a non-hardened or crosslinked form, should not be subjected to temperatures in excess of 100°C, as such heat sterilization tends to destroy the gel structure. It is, therefore, necessary in utilizing the filters for biological processes to prepare them sterilely from the beginning. On the other hand, it has been found possible to harden or crosslink the filter during the preparation by carrying out the gel formation in the presence of Factor FXIII and calcium ions. Where Factor FXIII is to be present, it is preferably present in an amount of at least 5 units per gram fibrinogen, preferably at least 50 units per gram. Calcium is present in a concentration of at least 20 mM.

A still stronger filter is obtained by effecting crosslinking with one of the above-mentioned crosslinking agents, especially a dialdehyde and particularly one of the formula OCH-R-CHO, wherein R is an alkylene group of 1 to 8 carbon atoms, such as glutardialdehyde. The filter obtained in this way can be heat treated in an autoclave and consequently sterilized.

The clotting parameters are above all the enzyme concentration, e.g. between 100 and 3000 NIH units/L for throm-

bin and for the fibrinogen concentration between 0.1 and 70 g/l, preferably between 1 and 10 g/l, increased concentration giving a tighter gel. A tighter gel has a smaller pore size. Increased ionic strength also provides a tighter gel as well as a higher pH. It is preferred to carry out the gel preparation using a gel mixture having a pH of between 5.5 and 11, preferably between 6 and 9, and an ionic strength between 0.05 and 0.5. Gels formed at calcium ion concentrations between 0 and 20 mM are tighter.

Pore size is also affected by the temperature at which the clotting (gelation) is effected. A lower temperature of gelation means an increased clotting time, which in turn means that the resultant gel has a larger pore size. As a result of its larger pore size, it provides a greater rate of flow.

The gel of the invention can be used other than as a filter. One can dispose catalytically active substances such as catalytically active enzymes or catalytically active metals within the pores and thus use the pores' structure as a catalyst. The filter, therefore, can act more or less as a catalyst support for the catalytically active agent disposed therein. When the catalytically active agent is disposed within the pores, the resultant structure can be employed as a size selective catalyst converting only those components whose size is such as to freely pass through the pores of the catalyst support. Those materials retained in the surface of the gel are not catalytically converted.

By such a filter, one can conveniently effect enzymatic conversions, especially when the enzyme is immobilized

within the filter covalently, ionically or otherwise. Since the gel structure is formed by the use of an enzyme, the filter of the invention's chemical components is compatible with the enzyme being employed as an enzyme catalyst. Thus, one can use the filter of the invention for any of the following enzyme conversions when the same contains the appropriate enzyme to effect that enzymatic catalysis: for reactions involving various oxido-reductases, transferases, hydrolases, lyases, isomerases and ligasis (synthetases). Hydrolases which have capacity of degrading the protein strands in the gels cannot be used.

The method by which the enzyme or other catalytic component is disposed within the filter, i.e., within the pores of the filter, depends upon the nature of the enzyme. Preferably it is disposed by the use of a known enzyme immobilizing agent followed by washing of the filter to remove extraneous materials.

One can also dispose reactive cellular components within the pores of the gels. Upon reaction, low molecular weight components may be released and subsequently eluted from the gels. An example of such a type of reaction is production of interferon by leukocytes after their reaction with Sendai virus. As shown in this invention, both of these components can be disposed within the pores of the gels.

## BRIEF DESCRIPTION OF DRAWINGS

The invention is described more in detail with reference to the enclosed drawing, in which:

Fig. 1 a shows molding of a filter according to the invention;

Fig. 1 b shows the filter arranged for filtering;

Figs. 2 a and 2 b show graphs of the flow as a function of the coagulation time at different pH with thrombin and Batroxobin, respectively;

Fig. 3 shows the turbidity of the fibrinogen solution (fibrinogen) as a function of the time after addition of thrombin;

Figs. 4 a and 4 b show the flow as a function of the coagulation time at different ionic strength of thrombin and Batroxobin, respectively;

Figs. 5 a and 5 b are graphs showing the relationship between protein concentration in the gel forming system and flow-rate.

Figs. 6 a and 6 b show the temperature plotted against the coagulation time and the flow, respectively, as a function of the coagulation time at different temperatures;

Fig. 7 shows the turbidity of the effluent (turbidity in the effluent) in % as a function of the pore diameter in µm;

Figs. 8 a, 8 b and 8 c are graphs plotting activation of fibrinogen and clotting time (Ct).

In order to more fully illustrate the invention and the manner of practicing the same, the following examples are presented.

EXAMPLE 1

METHODS AND MATERIALS

Human fibrinogen , Fraction 1-4 (7) was obtained from IMCO, Stockholm, Sweden. The preparation, either a freeze-dried powder or a wet paste, was 97-100% clottable (as determined spectrophotometrically). A solution being 0.3 M in NaCl and 2% in protein was prepared. This solution (50ml) was dialyzed against 0.3 M NaCl at 4° for 3 hrs. with changes of outer fluid (5 litre) every hour. This dialyzed solution was further diluted with deareated Tris-imidazole buffer (8) of pH between 6.5 and 8.2 to protein concentrations between 1.2 and 5.0 g/1. In the final dilutions the concentration of each Tris and imidazole was 0.02 M. When necessary increase in ionic strength was achieved by inclusion of sodium chloride in the buffer. In order to inhibit any trace of plasmin which may be generated, Trasylol (Bayer AG, Germany) was added to a concentration of 5 KIE/ml to all buffers and dialysis fluids.

In the gelation experiments the following procedures was employed: To 3.65 ml of fibrinogen solution in a plastic tube was added 70 µl of 1 M $CaCl_2$ solution, immediately followed by 50 µl of thrombin or Batroxobin solutions of varying concentrations. This mixture is called Reaction Mixture. The tubes are rapidly inverted twice and transferred to the gel cup or to the spectrophotometer cell within 10 seconds after addition of enzyme. The further handling is described under separate paragraphs.

Thrombin. In most experiments a bovine preparation prepared as previously described (9) was used. Specific activity: 100-200 NIH units per mg. Control experiments with highly purified (specific activity: about 2000 NIH units per mg human thrombin (10) was performed in some instances.

Batroxobin (from Bothrops marajoensis) was obtained from Pentapharm AG, Basel, Switzerland. Specific activity: 505 BU per mg.

Hirudin was also obtained from Pentapharm AG. Specific activity: 1000 ATU per mg.

Reagents. All reagents used were of analytical grade.

## PREPARATION OF GEL COLUMN

A solution of thrombin is added to a solution of fibrinogen in a tris-imidazole buffer containing calcium salts with a pH of between 6.5 and 8.2 and an ionic strength between 0.1 and 0.3 so that the final concentration is between 0.05 and 2.5 NIH-units per ml. In other tests Batroxobin is used to obtain gel formation in a concentration between 0.27 and 3.6 BU per ml. The concentration of "Tris" and imidazole salts is each 0.02 M and the concentration of calcium salt is also 0.02 M. The variation in ionic strength is obtained by addition of NaCl.

Gels are also prepared at calcium ion concentration between 0 and 20 mM. With reduced calcium ion concentration the opacity of the gels is increased. When thrombin is used to achieve the gel formation the clotting time (Ct), also in the absence of calcium ions is directly proportional to the flow rate and thus also to $K_s$. When "Batroxobin" is used for the gel formation, the

stability of the gels in the absence of calcium ions is unsatisfactory, which makes flow measurements more difficult.

After addition of an enzyme such as thrombin or Batroxobin, the solution is rapidly mixed and then poured into a cup, e.g., such a one shown in Fig. 1a. It is made of acrylic plastic*) and has an inside diameter of about 14 mm and a height of about 27 mm. The plastic cup is shown in Fig. 1a and the lower part of the cup is provided with a nylon filter having a mesh size of 80 x 80 µm. This filter is fastened by a plastic guard ring. A film layer, e.g., "Parafilm ®", is preferably applied at the lower portion so that liquid is prevented from leaking out of the cup. Immediately after introducing the solution into the cup, a silk net with the mesh size 150 x 180 µm is adapted at the upper end and is fastened with a guard ring. The liquid in the gel cup can, e.g., be about 1 mm over the net surface. The cup is left at room temperature for at least 2 hours for complete gel formation, preferably in a place free of vibrations.

After this time, the film is removed at the lower portion of the cup and with its contents of gel is placed in the holder A according to Fig. 1b, a holder B is applied over the upper end of the cup 1. At the upper end of the holder B there is an opening as well as at the lower end of the holder A. The holder B is filled with liquid (buffer or water) and a rubber cork provided with a tube, which is connected with a rubber hose is inserted into the opening. The rubber hose is connected with a container for permeation solution which is allowed to fill the rubber hose without air bubbles. The container (not shown in the drawing) is placed at such a height that a suitable flow is

*) (other materials can also be used such as nylon and pol-

obtained through the gel. The hydrostatic pressure is varied at different tests between 4 and 40 x $10^3$ dynes/$cm^2$. (4 a 40 x $10^{-2}$ N/$cm^2$).

The fibrinogen used for the preparation of the gels contains trace amounts of factor XIII, which is a transamidase. In the presence of this enzyme and calcium ions, covalent intermolecular cross-linkages between chains in the molecule units of the fibrin gel are formed. This is especially the case when thrombin is present as thrombin activates factor XIII.

An electrophoretic analysis of reduced fibrin from various gels in the presence of sodium-dodecyl sulphate shows that a complete cross-linking of the $\alpha$ and $\gamma$ -chains of the fibrin takes place in the presence of thrombin. A partial cross-linking takes place in the presence of Batroxobin. The covalent cross-linkings formed in the presence of factor XIII contributes to the stabilization of the gel structure.

The silk net applied to the top of the gel cup and which is in intimate contact with the gel matrix is of great importance for the mechanical stability of the gels. Without this net or some other means for preventing collapse of the gel, the gel compound is destroyed in the flow tests, the gel collapsing in the central protion and a conical inward bend arising.

The silk net can, of course, be replaced with other nets, e.g., of cotton, nylon, iron or copper, which also stabilize the gel structure at pressures up to 40 x $10^3$ dynes/$cm^2$. (4 x $10^{-2}$ N/$cm^2$)

Turbidity measurements

In parallel to the flow studies, the turbidity profile of the system was determined under identical conditions. In these experiments the Reaction Mixture (see under Fibrinogen) was

poured into a cuvette (5ml) of a recording spectrophotometer (Beckman Acta III) and the turbidity (optical density) recorded at 450 nm. After a lag-phase there was a rapid increase in turbidity (cf. Fig. 8) which was accompanied by gelation. A tangent was drawn to the steepest part of the sigmoidal curve. Its intersection with the time axis is defined as the gelation or clotting time (Ct). (Ct is about the same as the time for visually observed turbidity increase in the gel cup.) In addition to Ct also maximum turbidity (OD-max) and rate of turbidity increase ( Δ OD/min) was recorded. The time required for gelation to reach completion was judged from the turbidity curve. This time reanged from 1 hr. to 2 hrs. for the high and the low enzyme concentrations, respectively.

Determination of fibrinopeptides and cross-linking

Reaction Mixtures (see under Fibrinogen) were prepared in several identical tubes. One of them was used for turbidity measurement as described above. The other tubes contained each 1 ml of Reaction Mixture. The reaction in the latter tubes was quenched at different times by addition of hirudin (2 ATU/ml) and an equal volume of 8 M urea. Thereafter the fibrin (ogen) was precipitated by addition of an equal volume of chilled ethanol. The mixtures were kept on ice-bath for 2 hrs. and thereafter the precipitates were secured by centrifugation, dissolved in urea and used for SDS-gel electrophoresis. The supernatants were used for radioimmunoassay (RIA) of FPA, FPB and B β 15-42 was assayed using the recently developed method of Kudryk et al.

Viscosity was determined with a viscometer type Ubbelohde, having a flow time for water of about 290 sec at 25°C. It was calibrated against a standard (CNI. Cannon Instrument Company, Pa. USA). Density was determined with a 5 ml pycnometer.

Pore size. The equation for calculation of average pore size of membranes (18) and acrylamide polymer gels (4) was applied:

$$r = \sqrt{\frac{8Ks}{\varepsilon}} \quad (2) \quad (3)$$

where r is the average pore radius (in cm), and $\varepsilon$ is the fractional void volume of the gel, i.e., the fractional volume of liquid in the gel. $\varepsilon$ is calculated on the basis of protein concentration assuming a partial specific volume for fibrinogen of 0.72 (19), $\varepsilon$ is in this case the fractional void volume for gels in which no water is bound to the gel matrix. However, the degree of hydration of fibrinogen in solution has been reported as high as 6 g per g protein.(20). Assuming that this water is retained by the gel matrix we also calculated $\varepsilon$ for such hydrated gels.

Diffusion coefficient. The apparent diffusion coefficient of water in the gel was calculated from Ks according to Ticknor (21) and White (4).

$$D = \frac{R.T.Ks}{\varepsilon .V. \eta} \quad (3) (5)$$

where D is the diffusion coefficient (in $cm^2$/sec.). R is the gas constant (in ergs/mole-degree), T is the absolute temperature (in °K) and V is the molar volume of the permeant (in $cm^3$/mole).

Ionic strengths were calculated on basis of the molarity of the electrolytes. Activity coefficients and degree of calcium binding to protein were not taken into account.

Least square analysis was used for calculation of correlation coefficients, slopes and intercepts. All lines shown in figures were drawn accordingly.

## RESULTS

### Preparation and stability of gels

The flow studies were performed on gels which had been formed at ambient temperature. The average temperature was 24 ± 2°C. However, in each series of experiments the variation in temperature never exceeded 2°C. Preliminary experiments suggested that this variation in temperature has a negligable effect on the Ct of the system. In the permeation experiments, when not otherwise stated, the flow-rates were corrected to 25°C.

The silk net at the upper end of the gels stabilizes the gel structures. Without support of the silk net, the gels will yield to flow at the pressure applied (about $7 \times 10^3$ dynes $/cm^2$). The yielding is only noted at the center of the gel, since the gel matrix adhers firmly to the walls of the plastic cup.

The nets in the column do not significantly reduce the flow-rate of liquid in columns without gels. We, therefore, assume that also when the nets are in contact with gels they do not restrict the area available for flow.

Before a flow experiment was started, the extent of incorporation of fibrinogen into the gel matrix was determined. This was done by determining the protein content in the void

volume of the column (about 4 ml). The amount of protein, as measured spectrophotometrically using the extinction coefficient of fibrinogen (22), ranged between 1 and 3% of the total protein used for gelation. When deemed necessary the non-clottable portion was taken into account in calculations of the fibrin content of gels.

The effect of changing permeant on the flow-rate of gels was studied in some experiments. A representative series of experiments is shown in Table I. The gel was first percolated with buffer of ionic strength 0.21 (experiment I). On changing the permeant to water (experiment II) an increase in flow-rate occurred, which is larger than expected on the basis of the viscosity change of the permeant. On return to the original permeant (experiment III) the flow-rate decreased, but not completely to the original value (experiment I). When buffer of ionic strength 0.36 was percolated through the gel (experiment IV) a small decrease occurred which was almost as expected on the basis of the difference in viscosity between the two buffers. When the permeant was again changed to water (experiment V) the flow-rate increases to almost the same value as after the first change to water (experiment II). These results suggest that the final gel structure is not influenced by moderate changes in permeant composition, but changes may occur on drastic changes in ionic environment and these are not completely reversible.

TABLE I 26

Flow Properties of Fibrin Gels with Different Permeants.

Gel formation: Tris-imidazole buffer pH 7.4, ionic strength 0.21, thrombin 0.8 NIH-units/ml, temperature 21°C and fibrinogen concentration 2 mg/ml.

Permeation: $H_2O$, at 22°C - 23.5°C.

| Experiment | Permeant | Flow, ml/hr | % |
|---|---|---|---|
| I | Tris-imidazole pH 7.4, $\Gamma/2$ 0.21 | 3.177 | 100 |
| II | $H_2O$ | 3.708 | 117 |
| III | Tris-imidazole pH 7.4, $\Gamma/2$ 0.21 | 3.385 | 106 |
| IV | Tris-imidazole pH 7.4 $\Gamma/2$ 0.36 | 3.271 | 103 |
| V | $H_2O$ | 3.649 | 115 |

Flow pattern through fibrin gels

Viscous flow. In order to test if the flow obeyed Poiseuille's law, the flow-rate at different pressures (4.5 - 5.6 x $10^3$ dyne/$cm^2$) for gels formed at pH 7.4, ionic strength 0.21 was determined, at three different thrombin concentrations (0.1-0.8 NIH units/ml). The Ks-range for these gels was $10^{-8}$ to $10^{-10}$. Permeation was in one experiment with the same buffer as above and in the other cases with water. In all cases the drop-rate decreased linearly with decreasing pressure. As shown in Table II for one of the gels, the flow-rate per unit pressure was almost independent of total pressure.

In another series of experiments the flow-rates were determined with permeants of different viscosities. The gels used

<(4.5-5.6x $10^{-2}$ N/cm²)>

in these experiments were formed at pH 7.4, ionic strength 0.21, at four fibrinogen concentrations. Thrombin as well as Batroxobin were used as inducers of gel formation. Permeation was performed at five different temperatures between 4.5°c and 40°c. In all cases there was a linear relationship between the inverse viscosity of the permeant and the flow-rate. These experiments suggest that the flow through the gels is viscous. In addition Reynold's number was calculated and found to be within the laminar region for all gels.

Diffusive flow. It was pointed out by Ticknor, J. Phys. Chem 62, 1483-5 (1958) that the equation for viscous flow is identical in form to equations for diffusive flow, when the relationship between diffusion coefficient (D) and viscosity according to Johnson and Babb, Chem. Revs. 56, 387-453 (1956) is taken into consideration The relation between Ks and D is given in Equation 5. In flow experiments using water as permeant we calculated the apparent diffusion coefficient for water at 22°c- 23°c. Even for the tighest gels ($Ks \approx 10^{-10}$), the calculated D-values were 6-orders of magnitude larger than the reported self-diffusion coefficient of water at 25°c ($2.8 \times 10^{-5}$ $cm^2$/sec.). This supports the above conclusion that the flow through the fibrin gels is predominately viscous.

TABLE II

Relationship Between Pressure and Flow-Rate.

Gelformation: pH 7.4, ionic strength 21, thrombin 0.1 NIH unit/ml temperature 23.5°C and fibrinogen concentration 2 mg/ml.

Permeation: $H_2O$, temperature 23.5°. Ks = $9 \times 10^{-9}$

| Pressure, dyne/cm$^2$ <-> | % | Flow ml/hr | Flow per dyne per cm$^2$ <-> ml/hr x $10^3$ | % |
|---|---|---|---|---|
| 5531 | 100 | 11.280 | 2.0394 | 100 |
| 5319 | 96.2 | 10.817 | 2.0337 | 99.7 |
| 5127 | 92.7 | 10.418 | 2.0320 | 99.6 |
| 4874 | 88.1 | 9.859 | 2.0228 | 99.2 |
| 4576 | 82.1 | 9.148 | 1.9991 | 98.0 |

Gel Permeability and clotting time (Ct)

There is a correlation between clotting time (Ct) of fibrinogen and enzyme concentration. We explored the relationship between Ct and permeability of the final gels. Therefore, at the same time as gels were prepared for permeability studies, the Ct of the gel forming system was determined in parallel experiments by turbidity measurements (see Methods).

pH. At a constant fibrinogen concentration and ionic strength, the flow-rates for both thrombin and Batroxobin gels were directly related to the Ct of the gel forming system over a wide range of Ct (17 sec - 500 sec). This applied to three different pH's (6.5, 7.4 and 8.2) as exemplified in Fig. 2. At all pH's there was a difference in slope between curves for thrombin as compared to those for Batroxobin.



< (1 dyne/cm$^2$ = $1.0 \times 10^{-5}$ N/cm$^2$) >

At each pH, the correlation coefficients (r) for six different Ct versus flow-rate curves (4 experimental points in each) were calculated. The mean r-values and their standard deviations (SD) were as follows: at pH 6.5, 0.9709 $\pm$ 0.0184; at pH 7.4, 0.9721 $\pm$ 0.0394; at pH 8.2, 0.9599 $\pm$ 0.0434. There was no significant difference in r-values for thrombin and Batroxobin curves.

Ionic strength. In another series of experiments the ionic strength of the gel forming system was, at constant protein concentration, varied between 0.21 and 0.31. At all pH's (6.5,7.4, 8.2) an increase in ionic strength from 0.2 to 0.3 resulted in a decrease in flow-rate by roughly one order of magnitude. This applied to both thrombin and Batroxobin gels.

Ct were prolonged with increasing ionic strength at all enzyme concentrations and pH's. At each ionic strenth, however, there was for both thrombin and Batroxobin gels a linear relationship between Ct and flow-rate. The results at pH 7.4 is shown in Fig. 4. At all ionic strengths a difference in slope between curves for thrombin as compared to those for Batroxobin was noted.

At two ionic strengths, regardless of pH, r-values for six different Ct versus flow-rate curves (4 experimental points in each) were calculated. Mean r-values and SD were as follows: at ionic strength 0.21, 0.9851 $\pm$ 0.0208 and at ionic strength 0.26, 0.9511 $\pm$ 0.0470. There was no significant difference in r-values for thrombin and Batroxobin curves.

Gel permeability and fibrinogen concentration

In a series of experiments we showed that the relationship between Ct and flow-rate applied to a wide range of protein concentrations in the gel forming system. These experiments were only performed at pH 7.4 and ionic strength 0.21. When Ct at a given protein concentration were plotted against flow-rates a linear relationship, similar to that shown in Fig. 3 (pH 7.4), was demonstrated at all fibrinogen concentrations (1.5 - 5.0 g/l) The plots for both thrombin and Batroxobin converged towards an intercept near the origin with decreasing clossing times. Like in the experiments shown in Fig. 2, the slopes for Batroxobin curves were steeper than those for thrombin at all protein concentrations. The r-values for 8 different Ct versus flow-rate curves (8 points in each) were calculated. Mean r-values and SD were as follows: for thrombin, $0.9800 \pm 0.0157$ and for Batroxobin, $0.9830 \pm 0.0187$.

Table III shows Ct at different protein and enzyme concentrations in one series of experiments. In case of Batroxobin, increasing protein concentrations did not markedly influence Ct. However, in the case of thrombin there is a small prolongation of Ct with increasing fibrinogen concentrations.

The relationship between protein concentration in the gel forming system and flow-rate was next studied. Fig. 5 shows the result of one series of experiments. It is evident that there exists, at different enzyme concentrations, a linear relationship between flow-rate and inverse protein concentration. The curves for thrombin and Batroxobin gels converge to a more or less common intercept near the origin with increasing protein con-

centration. The r-values for 15 different 1/C versus flow-rate curves (4-8 experimental points in each) were calculated. Mean r-values and SD (standard deviation) were as follows: for thrombin, 0.9738 ± 0.0308 and for Batroxobin, 0.9711 ± 0.0356.

It is apparent from Fig. 2 how the flow rate $\frac{Q}{t}$, at a constant hydrostatic pressure, is directly proportional to the coagulation time (Ct) of the thrombin-fibrinogen mixture at different pH. The coagulation time of the system is determined spectrofotometrically in a separate test under otherwise identical conditions. The optical density OD at 450 nm is determined. At gel formation the turbidity of the solution increases rapidly as shown in Fig. 3. The tangent of the steepest portion of the curve intersects the time axis at a distance designated as coagulation time Ct. As there is a direct relation between Ct and flow rate $\frac{Q}{t}$, Ks is also directly correlated with Ct according to equation 1.

It is apparent from Fig. 4 that the flow rate of gels formed at different ionic strength is always directly correlated to the Ct of the enzyme-fibrinogen solution used in the gel preparation. In addition the great influence on the flow rate at a change of the ionic strength is pointed out.

As is apparent from Fig. 5, the flow rate $\frac{Q}{t}$ is inversely proportional to the fibrin concentration (fibrinogen concentration) in the gel. Thus, according to equation 1, Ks will also be inversely proportional to the fibrinogen concentration.

The flow is dependent on the temperature, as according to equation 1, the flow is inversely proportional to the viscosity of the permeation solution. The temperature in gel formation is

also of importance as a constant enzyme concentration, Ct is reduced at a higher temperature. This is apparent from Fig 6a. However, the flow rate in gels formed at different temperatures is directly proportional at Ct at the relative temperature, as is evident from Fig. 6b.

The columns prepared in the way schematically illustrated in Fig. 1 are of small dimensions (1.5 $cm^2$ x 2.6 cm). Similar qualitative results are observed with gel columns of greater dimensions (5 $cm^2$ x 12 cm). When nothing else is indicated, the smaller type of column is used in the tests.

Example 2

Standardization of pore size with latex particles of a known size.

Spherical latex particels of diameters between 0.085 ± 0.0055 (SD) µm (SD = standard deviation) and 0.198 ± 0.0036 (SD) µm from Dow Chemicals, USA, were used in the tests. A number of gels formed at pH 7.4 and at two different ionic strengths, were used. In the tests, Ct varied from 23 to 314 seconds. The theoretical radius was calculated for each gel according to equation 3 assuming that a cylindrical vertical capillary system was present.

Fig. 7 shows two series of tests. In series I the ionic strength was 0.23 during the gel formation and in series II 0.21. The gel columns were equilibriated with water. After this, suspensions of particles were applied to the gels. In series I the particle size was 0.085 µm and in series II 0.198 µm. The particles were slurried in water to a concentration of 0.1% (weight/volume). The turbidity (at 450nm) of the effluent was determined. It was then possible to establish by means of the turbidity values if the latex particles had passed the filter.

In Fig. 7 the turbidity has been expressed in % of maximum turbidity of the effluent. As is apparent from Fig. 7 the turbidity of the effluent increases above a certain theoretical pore size of the gel. At an additional increase in pore size more particles will pass through the gel (filter) and over a certain pore size a constant amount of particles permeate the gels. The difference in theoretical pore size between no and

complete permeation is a measure of the sum of pores and particle variation. The pore size at 50% permeation is an expression of the average size of pores and particles. If the total variation in pore size is within the range of the average particle size $\pm$ 3 SD it can be assumed that the pore size in the gel is uniform.

In Fig. 7 the variation in particle size (average size $\pm$ 3 SD) has been shown with a horizontal line 50% permeation. It is apparent that the total variation can, to a large extent, be explained by the particle variation. It can be concluded from this that the pores in the gels are rather uniform. It is also apparent from Fig. 7 that the theoretical average pore size is about of an order (one ten power) greater than the real effective particle size. Thus, calculation of the pore size according to equation 3 only gives relative values for the pore size.

EXAMPLE 3

Passage of proteins and dextran trough fibrin gels

Various protein solutions were (applied) to fibrin gels prepared in the way described in Example 1. The gel formation was carried out at room temperature (21° - 25°C). In most cases, the buffer used in gel formation had the same composition as the buffer used for permeation. The filtration tests took place at room temperature (22° - 25°C). When nothing else is indicated, the volume of the gels was 1.47 $cm^2$ x 2.48 cm = 3.65 ml. The tests were carried out on different days and with different fibrinogen preparations. Thus, it is not possible to make a comparison as to the pore size between different tests. Table 1 shows the proteins tested with respect to filtering ability through fibrin gels of different porosities. It is apparent from the table, that proteins, including those having a very high molecular weight, are filtered even through gels having small pore sizes. A high molecular weight polysaccharide ("Blue Dextran") shows the same filtration properties as the proteins. The table shows that the yield of proteins in the eluate is high, from which it appears that at least at room temperature the interaction between gel matrix and proteins is small. This also applied to such proteins as fibrinogen, fibronectin and the factor VIII complex.

## TABLE I

Filtration of proteins and particles through fibrin gels

| Test | Diameter or mol. weight | Permeation Buffer, | Sample ml | Yield % | a) Pore diam. µm | Gel formation Ct s | Protein g/l | $\frac{\Gamma}{2}$ | pH | Enzyme |
|---|---|---|---|---|---|---|---|---|---|---|
| Human serum | - | TI buffert + $Ca^{2+}$ | 0.1 | 98.2 | 2,12-4,86 (0,125-0,286) | 16-230 | 3,42 | 0,21 | 7,40 | T |
| Human plasma | - | TI buffert -$Ca^{2+}$ | 0,1 | 101.1 | 0,288 | 260 | 4,56 | 0.26 | 7,40 | T |
| Fibrinogen (4g/l) | 340000 | TI buffert -$Ca^{2+}$ | 1,0 | 95,8 | 4,34 (0,255) | 124 | 2,157 | 0,21 | 7,40 | T |
| Fibrinogen (4g/l) | 340000 | TI buffert +$Ca^{2+}$ | 1,0 | 97.8 | 2,33 (0,137) | 66 | 2,157 | 0,21 | 7,40 | B |
| Fibronektin (2,3g/l) | 440000 | TI buffert -$Ca^{2+}$ | 0,4 | 109 | 2,30 (0,135) | 430 | 2,274 | 0.21 | 7,40 | T |
| Antihemophilic factor (FVIII)b | 2-6x $10^6$ | TI buffert +$Ca^{2+}$ | 1,0 | 99 8 | 2,48 (0,146) | | 3.000 | 0.21 | 7,40 | T |
| | | | 1.0 | 63 | 1,08 (0,064) | | 5,000 | 0,26 | 7,40 | |
| Ferritin (15g/l) | 2x$10^6$ | TI buffert +$Ca^{2+}$ | 0,2 | 103,8 | 0,276 (0,016) | 440 | 4,56 | 0,26 | 7,40 | T |

a) The theoretical diameter was calculated according to equation 3. The effective diameter through calibration with latex particles.

The effective pore diameter is given within brackets. (Based on a ratio $\frac{\text{theoretical}}{\text{effective}}$ of about 17)

b) The volumes of the gel column; 4,9 cm x 11 cm = 53.9 ml.

TABLE 1 (cont.)

| Test | Diameter or mol. weight | Permeation Buffer | Sample ml | Yield % | a) Pore diam. µm | Gel formation Cts | Protein g/l | $f/2$ | pH | Enzym |
|---|---|---|---|---|---|---|---|---|---|---|
| Fibrinogen (1 g/l) | $3 \times 10^6$ | TI buffer x $Ca^{2+}$ | 0.2 | 97.7 | 0.276 (0.016) | 440 | 4.56 | 0.26 | 7.40 | T |
| Normal human red blood corpuscles $5 \times 10^5/\mu l$ | 7 µm | Physiological common salt solution $-Ca^{2+}$ | 0.1 | 0 | 3.60 (0.212) | 255 | 1.66 | 0.21 | 7.40 | T |
| Human Platelets ($4 \times 10^5/\mu l$ plasma) | 2 ~ 4 µm | TI buffer $-Ca^{2+}$, +10m M EDTA | 0.3-5 | 0 | 4,86 (0.286) | 228 | 3.42 | 0.21 | 7.40 | T |
| Rat liver mitochondria | 0.5 µm | TI buffer $-Ca^{2+}$ +0.25M sykros, +10m M EDTA | 0.3 | 0 | 4.40 (0.259) | 524 | 2.16 | 0.21 | 7.40 | T |
| E. Coli | 0.8x1,2 µ | TI-buffer $-Ca^{2+}$ | 45 | 0 | 1.78 (0.105) | 24 | 5.00 | 0.21 | 7.40 | T |
| Sendai-virus (640) hämagglutination units /ml | 0.15 µm | TI buffer $-Ca^{2+}$, + 1% BSA | I 0.2 | 0 | 0.284 (0.017) | 33 | 4.63 | 0.233 | 7.40 | T |
| | | | II 0.2 | 50.3 | 2.36 (0.139) | 19 | 2.08 | 0.21 | 7.40 | T |
| | | | III 0.2 | 95.2 | 4.46 (0.262) | 137 | 2.08 | 0.21 | 7.40 | T |
| Sendaivirus filtered through hardened and autoclaved thin layer fibrin gel (see Example 6 and Table 2-III) | 0.15 µm | TI buffer $+Ca^{2+}$ | 5 | - | 0.268 (0 016) | 60 | 9.86 | 0.26 | 7.40 | T |
| | | in the filtrate | | 0.86 | | | | | | |
| | | - on the filter | | 85.0 | | | | | | |

EXAMPLE 4

Filtration of suspensions of red blood corpuscles through fibrin gels

Fibrin gels were prepared in the way described in Example I and the conditions of gel formation is shown in Example 3. A small amount (0.2 ml) of human blood was applied to a gel column. Continued filtration was carried out at room temperature (22°-25°C) under the conditions shown in Example 3. The blood corpuscles did not pass through the fibrin gel. This was expected as the diameter of the red blood corpuscles (7-8 µm) is much larger than the effective pore diameter of the fibrin gel.

EXAMPLE 5a

Filtration of plasma rich in platelets through fibrin gels

Plasma rich in platelets (PRP) was prepared from blood by centrifugation for 4 minutes at 120g, the blood being drawn in citrate solution to prevent coagulation. It was cetrifugaeed at 2000 g for 5 minutes to remove the remaining red blood corpuscles and EDTA at a concentration of 10 mM was added to the PRP. 0.5 ml of the PRP was applied to a fibrin gel column prepared in the way described in Example I. The conditions of gel formation is shown in Example 3 and filtration was continued under the conditions shown in Example 3. To prevent aggregation of the platelets and their adhesion to the gel matrix, EDTA (10 mM) was added not only to PRP but also to the solution which was filtered. No platelets could be demonstrated in the eluate from the fibrin gel column. This was expected as the diameter of the platelet lies between 2 and 4 µm, which is considerably more than the effective pore size of the gel.

EXAMPLE 5b

Separation of mitochondia from fragments of liver cell by filtration through fibrin gels

Liver cells of a rat were homogenized in a homogenizator according to Potter-Elvehjem. Separation of cell fragments was achieved by differential centrifugation in known manner. The mitochondria were slurried in a buffer solution containing Na-EDTA (10 $_m$M) and succrose (0.25M). 0.3 ml of the resulting suspension was applied to a fibrin gel column prepared in the way described in Example 1. The conditions of gel formation is shown in Table I and filtration was continued under the conditions indicated in Table 1. No mitochondria could be demonstrated in the eluate, which was as expected, since their diameter is about 0.5 µm, thus considerably bigger than the effective pore size of the gel.

Separation of Sendai-virus by filtration through fibrin gels

Sendai virus is a virus specific to mice which is used for preparation of interferon in human lymphocyte cultures. A partially purified virus preparation (640 hemagglutination units/ml) was used in the tests.

0.2 ml of the virus suspension was applied to each of three fibrin gel columns prepared in the way described in Example 1. The conditions of the gel formation appear from Table 1 and filtration was continued under the conditions shown in Table 1. No hemagglutination activity could be demonstrated in the eluate from column 1; 50% of hemagglutination activity were demonstrated in the eluate from column II and 95% of the hemagglutination activity of the virus particles was demonstrated in the eluate from column III (see Table 1).

After filtration the silk nets at the upper part of the three columns were washed with a buffer solution (containing 1 % of bovine serum albumin, BSA) and the hemagglutination activity of the washings was analyzed. In the washing liquid from columns 1 100% of the hemagglutination activity was found; in the washing liquid from column II 25% of the activity was found and in the washing liquid from column III no activity was found.

The particle diameter of Sendai virus is stated to be about 0.15 µm. The tests show that when the effective pore radius is more than 0.15 µm the virus particles pass through the gel. When the effective pore radius of the gel is less than 0.15 µm a retention of the particles will, on the other hand, occur.

EXAMPLE 7

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Separation of Eschericia Coli (E. coli) by filtration through fibrin gel.

E. coli is an elongated intestinal bacterium of the approximate dimensions 0.8 x 1-2 µm. A suspension of E. coli in tris-imidazole-buffer, free of calcium and with pH 7.4 and ionic strength 0.21, was prepared (see Example 1). The suspension contained between $10^7$ and $10^8$ bacteria/ml. 45 ml of the suspension were supplied to a fibrin gel column of the dimensions 5 $cm^2$ x 11 cm prepared in the way described in Example 1. The conditions of the gel formation and the filtration are shown in Table 1. The flow rate was 31 ml/h. No bacteria passed through the gel, determined by turbidity measurements of the eluate from the column. The flow rate at constant pressure was less at the end of the test than at its beginning. Assuming an unchanged $K_s$, the reduction of surface corresponding to the reduction in flow can be calculated according to equation 1. According to this calculation the surface had been reduced to 58%. Thus, one might expect that the bacteria were enriched on the upper gel surface. By washing the silk net attached to the upper part of the gel with buffer solution 99% of the bacteria applied to the gel were found in the washing liquid.

The test shows that E. coli cannot pass through gels having a pore diameter which is considerably less than the smallest diameter of the bacteria.

EXAMPLE 8



Preparation of gels with reinforcement of porous plastic

In the foregoing examples nets of silk, plastic or metal adapted to the upper and lower portions of the gel have served as stabilizing structure of the fibrin gels. A corresponding stability can also be obtained in such a way that the fibrin gel is cast into a porous plastic, e.g. polyurethane, polyester or some similar porous plastic material, preferably one which is wettable by water.

In this example a foam plastic of polyurethane ("Regilen 40 AG") of a pore size 0.4 mm has been used. The gels were cast in a special apparatus. This consisted of a cylindrical plastic chamber in which the porous plastic had been introduced; the plastic was accomodated in a ring of acrylic plastic (height 2 cm and diameter 9 cm). The apparatus (chamber) had an opening at the upper and lower end, respectively. One opening was connected to a vacuum pump and the other opening was kept closed. The chamber was evacuated by means of the vacuum pump. After this, the valve connecting the chamber with the vacuum pump was shut off. A fibrinogen-trombin solution was subsequently allowed to fill the chamber rapidly through the valve in the opposite opening. The valve was thereafter closed and the chamber was left for 2 hours, so that the fibrinogen solution in the porous plastic material should be completely converted to a fibrin gel. The clotting parameters of the thrombin-fibrinogen mixture was shown in Table 2. For comparison, a gel was also prepared in the way described in Example 1. In Table 2 the Ks -value of this latter

gel is also shown. After complete gel formation the chamber was opened and the plastic cake with fibrin gel( including its plastic frame) was taken out. It was transferred to a special filter chamber. The framed ring, in which the plastic material and the gel were accomodated, fitted tightly to the edges of the filter chamber through two O-rings. The upper lid of the chamber was provided with an inlet for the liquid to be filterd and a ventilating valve to let our the air above the gel surface. In the lower portion of the chamber there was an outlet for collecting the filtered liquid. A buffer solution with the composition shown in Table 2, was filtered through the gel cake. The Ks value was calculated according to equation 1 (Table 2). As is apparent from the table the Ks-value of the gel, cast in plastic, is of the same order as the gel prepared according to Example 1. The partial specific volume of the plastic material in the gel cake is 0.03 which means that the plastic matrix reduces the surface available for flow only to a small extent.

EXAMPLE 9

Preparation of gels in a cellulose matrix

Cellulose materials can also be used as reinforcing agent (supporting substance). In this example, a porous cellulose compound ("Wehex cloth") is used as reinforcing agent of the fibrin gel. It had a thickness of 0.2 - 0.3 cm. Circular pieces of a radius of about 3 cm were wetted with a thrombin-fibrinogen solution. The cellulose pieces then swelled to about double thickness. The partial specific volume of the swollen cellulose compound was 0.04. Immediately after swelling which lasted for about 2 - 4 seconds the pieces were placed on the filter disc of a Büchner funnel. Measures were taken so that the pieces fitted tightly to the edges of the funnel. The openings of the funnel were covered with "Parafilm" and the funnel was left at room temperature for 2 hours in order to obtain a complete fibrin formation in the pores of the cellulose. Buffer solutions, the composition of which is shown in Table 1, were filtered through the gels. The Ks-value of the gels which are cast in cellulose is of the same order of magnitude as control gels prepared without reinforcing substance.

EXAMPLE 10

Preparation of fibrin gels in thin layers for filtration

In this example it is shown that fibrin gels in thin layers with reinforcement only on the lower surface can be used for filtration. About 10 ml of fibrinogen solution in tris-imidazole buffer with pH 7.4 were mixed with a thrombin solution. The mixture was thereafter poured into a Petri cup the bottom of which was covered by a damp silk cloth. The cup was covered with a lid and was left for 2 hours for a complete gel formation. The thickness of the gel layer was 2 mm. The clotting parameters of the gel is shown in Table 2. The filter was thereafter attached to a "Millipore" filter support provided with a funnel. The funnel was filled with buffer solution and the flow rate was determined. As is apparent from Table 2, the Ks-value is of the same order or magnitude for a corresponding fibrin gel prepared according to Example 1. However, the filter showed in course of time gradually diminishing Ks-values, which presumably is due to compression of the gel matrix during the flow.

EXAMPLE 11

Stabilization of gels by treatment with dialdehyde

In this example it is shown that gels prepared according to Examples 1,8 and 10 can be stabilized by treatment with dialdehyde.

A. A gel prepared according to Example 1 was first equilibrated with water and then brought into equilibrium with 0.014 M phosphate buffer solution with pH 7.2 in 0.15 M NaCl (phosphate buffered saline solution PBA). 2 - 4 column volumes of a 1% glutaraldehyde solution were then allowed to filter through the gel in the course of 10 minutes - 2 hours.After this the gel was washed with several column volumes of PBS and then with water. The column was finally equilibrated with tris-imidazole buffer and flow measurements were carried out. The Ks-value is almost unchanged after treatment with glutar dialdehyde. After the flow measurements, the gel was taken out and treated for 72 hours with 8 M urea containing 1% of sodium dodecyl sulphate (SDS). The gel was then reduced with 1% dithiotreitol in a way known per se. Polyacrylamide gel electrophoresis in the presence of SDS showed in comparison with non-stabilized gels the absence of free fibrin chains (fibrinogen chains), which can be interpreted as a proof that glutar dialdehyde had cross-linked the chain units of the fibrin structure.

B. A gel prepared in porous plastic according to Example 1 was first washed with a tris-imidazole buffer solution free of calcium and was then brought into equilibrium with a 0.014 M

phosphate buffer solution with pH 7.2 in 0.15 M NaCl (PBS). Two column volumes of a 1% glutar dialdehyde solution were then passed through the gel cake (column) in the course of 10 minutes. The gel cake was then washed with several column volumes of PBS and then with water. Finally the column was brought into equilibrium with tris-imidazole buffer and flow measurements were carried out. These are shown in Table 2. As is apparent from the Table the $K_s$-value is only slightly changed after the treatment with glutar dialdehyde and is of the same order of magnitude as a gel prepared according to Example 1. The gel stabilized with glutar dialdehyde was then autoclaved at 120°C for 20 minutes at a pressure of 1.4 atm. After autoclaving the flow of buffer solution was again tested through the gel cake. As is apparent from Table 2, autoclaving has influenced the flow properties of the gel only to a small extent. Cracks in the gel would have caused drastic increase of the flow through the gel.

C. A fibrin gel prepared according to Example 10 was transferred to a cup with 500 ml water to remove buffer salts by diffusion. After 2 hours the gel was transferred to a cup with a new portion of water. After 2 hours the gel was transferred to a cup with 500 ml phosphate buffer solution with pH 7.2 in 0.15 M NaCl (PBS) and was left over night. The gel was then transferred to a Petri cup containing 50 ml of 1% glutar dialdehyde. After 2 hours the glutardialdehyde solution was exchanged for a new portion of the same liquid. After additional 2 hours the gel was transferred to a cup with water and washed in the way described above. After washing with water the gel was transferred to a cup with tris-imidazole buffer solution. After 2 hours the washing liquid was exchanged for a new portion and after addit-



$\langle (1.4 \times 1.013 \times 10^5 \text{ Pa}) \rangle$

ional 12 hours the gel was transferred to a "Millipore"-filter carrier with a funnel. As a comparison, measurements were carried out with a gel prepared in the same way except the treatment with glutar-dialdehyde. Directly after casting, this gel was transferred to a "Millipore" filter container for flow measurements. As is apparent from Table 2 the flow through the vulcanized filter was comparable with that through the non-vulcanized filter at the start of the flow measurement. However, at the end of the measuring period, the $K_s$-value of the nonstabilized filter had been reduced to a large extent which was not the case with the vulcanized filter. After the flow measurement the filters were sterilized through autoclaving of the filter (and the filter apparatus) at 120°C for 20 minutes at the pressure of 1.4 atm. Flow measurements were carried out after the heat treatment and the $K_s$-values are shown in Table 2.

No flow could be demonstrated through the nonstabilized filter. On the other hand the vulcanized filter showed $K_s$-values of the same order before as well as after autoclaving.

5 ml of a suspension of Sendai-virus were supplied to the vulcanized filter. Filtration was carried out by means of a water suction. When the liquid had passed the filter, additional 5 ml of buffer solution were passed through the filter. This was repeated twice. The filtrate was tested for hemagglutination activity. Inconsiderable hemagglutination activity could be demonstrated. The upper surface of the filter was washed with several portions of buffer solution. The washing liquid was opalescent and its hemagglutination activity corresponded to a yield of virus particles of almost 100%.

These examples show that the filters can be stabilized with a dialdehyde such as glutar-dialdehyde.

| | Type of filter | Permeation Buffer. | Pressure $\frac{dyn/cm^2}{(10^{-5}\ N/cm^2)}$ | $K_s$ $cm^2$ | Diameter µm | Ct, s | Gel formation fbg. konc. g/l | $\frac{m}{2}$ | pH | temp, °C |
|---|---|---|---|---|---|---|---|---|---|---|
| I | a Fibrin gel in foam plastic | $TI_{+Ca^{2+}}$ | - | $5,82x10^{-9}$ | 4,38 (0,258) | 171 | 2,478 | 0,21 | 7,4 | 23 |
| | b Vulcanization | " | - | $5,20x10^{-9}$ | 4,14 (0,243) | " | " | " | " | " |
| | c Vulcanization and sterilization | " | - | $1,39x10^{-9}$ | 2,14 (0,126) | " | " | " | " | " |
| | d Control | " | - | $3,15x10^{-9}$ | 3,17 (0,264) | " | " | " | " | " |
| II | Fibrin gel in cellulose sponge | $TI_{+Ca^{2+}}$ | 3430 | $0,6\_1,0x10^{-11}$ | 0,139-0,180 (0,008-0,011) | 60 | 9,864 | 0,26 | 7,4 | 24 |
| | Control | " | 29400 | $1,14x10^{-11}$ | 0,192 (0,011) | " | " | " | " | " |
| III | a Thin layer fibrin gel | $TI_{+Ca^{2+}}$ | 7350 | $1,0-2,5x10^{-11}$ | 0,180-0,284 (0,011-0,017) | 60 | 9,864 | 0,26 | 7,4 | 24 |
| | b Vulcanization | " | 7350 | $3,6x10^{-11}$ | 0,343 (0,020) | " | " | " | " | " |
| | c Vulcanization and sterilization | " | 7350 | $2,24x10^{-11}$ | 0,269 (0,016) | " | " | " | " | " |
| | d Vulcanization and sterilization | " | $9,86x10^5$ | $3,52x10^{-12}$ | 0,107 (0,0063) | " | " | " | " | " |
| | e. Vulcanization and sterilization | " | 7350 | $1,66x10^{-11}$ | 0,232 (0,014) | " | " | " | " | " |
| | f Control | " | 29400 | $1,14x10^{-11}$ | 0,192 | 60 | 9,864 | 0,26 | 7,40 | 24 |

T' = tris-imidazol buffer

See also explanation to Table I.

1. A fibrin gel containing therein at least one catalytically active substance.

2. A fibrin gel according to claim 1 wherein said gel has substantially uniform pore sizes and contains means for retaining the shape of at least one surface of the gel against deformation when contacted by a flowing medium.

3. A fibrin gel according to claim 2 wherein said means for retaining the shape of at least one surface of said gel is disposed on at least the upper surface of said gel.

4. A fibrin gel according to claim 3 wherein said means for retaining the shape of at least the upper surface of said gel comprises a foraminous sheet member or a foam.

5. A fibrin gel according to claim 1 wherein said fibrin gel is itself disposed in a porous-plastic material

6. A fibrin gel according to claim 1 wherein said fibrin gel is disppsed in a cellulose matrix.

7. A fibrin gel according to claim 5 wherein said porous plastic material is a porous plastic that is wettable by water.

8. A fibrin gel according to claim 7 wherein said porous plastic material is polyurethane.

9. A fibrin gel according to claim 7 wherein said porous plastic material is polyester.

10. A fibrin gel according to claim 1 wherein said fibrin gel has substantially uniform pores of a theoretical diameter of 0.003 to 1 µm.

11. A fibrin gel according to claim 10 wherein said ge has pores of a theoretical diameter of 0.009 to 0.3 µm.

12. A fibrin gel according to claim 1 wherein said catalyst is a catalytically active enzyme or a catalytically active metal.

13. A fibrin gel according to claim 12 wherein said catalyst is a catalytically active metal.

14. A fibrin gel according ro claim 13 wherein said cat alyst is a catalytically active enzyme.

15. A fibrin gel according to claim 14 wherein said enzyme is an enzyme selected from the group consisting of an oxido-reductase, a transferase, a hydrolase, a lyase, an isomerase, and a ligasis except a hydrolase which has the capacity of degrading a protein strand in a gel.

16. A fibrin gel having disposed therein a reactive celiular component.

17. A fibrin gel according to claim 16 wherein said fibrin gel contains leucocytes.

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FIG. 1a.

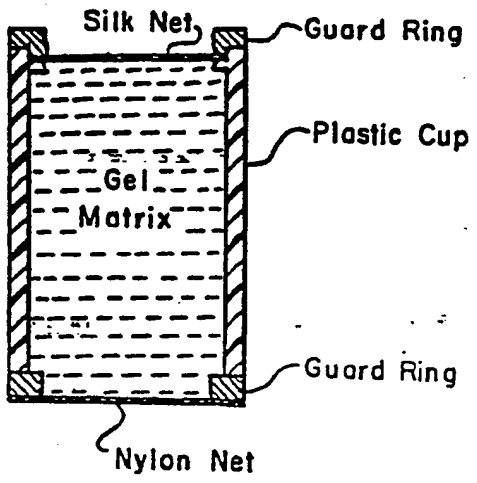



FIG. 1b.

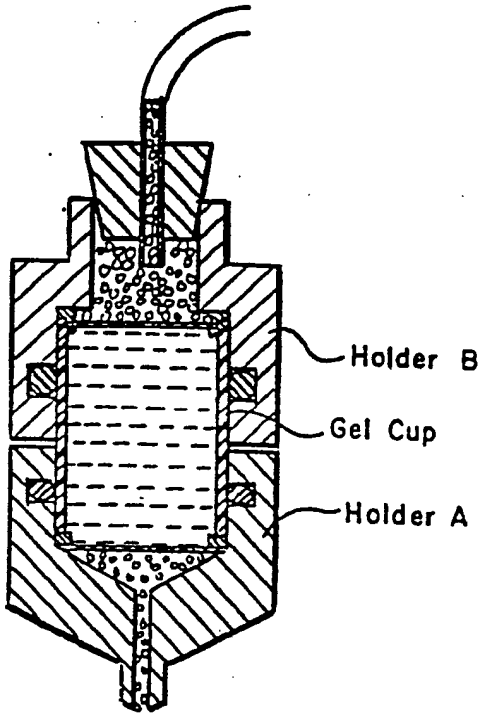



FIG. 8a.

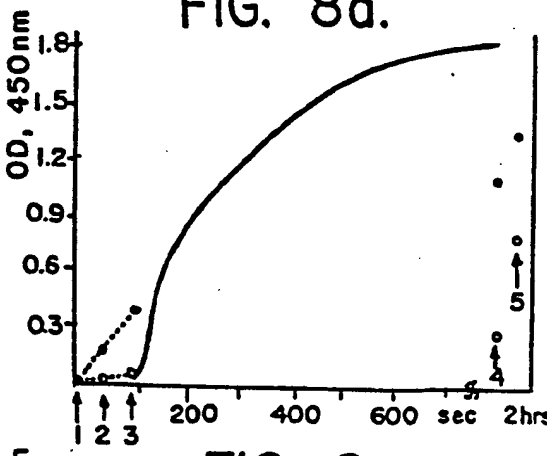



FIG. 8b.

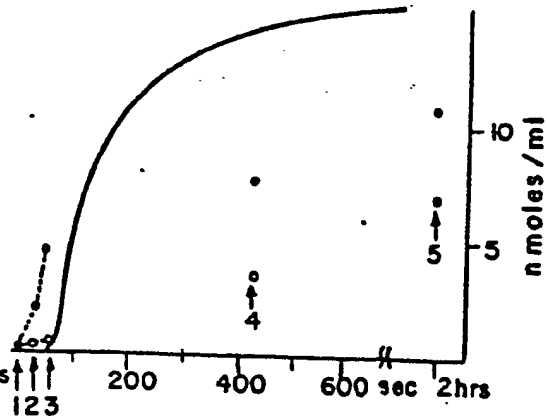
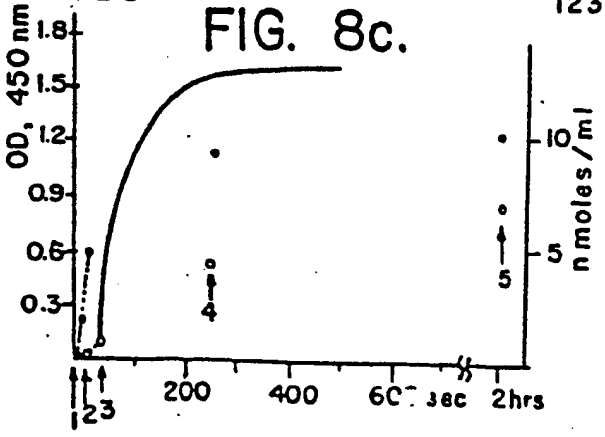



FIG. 8c.




Turbidity profile and release of fibrinopeptides during gelation

Optical density
FPA
FPB

FIG. 2b.

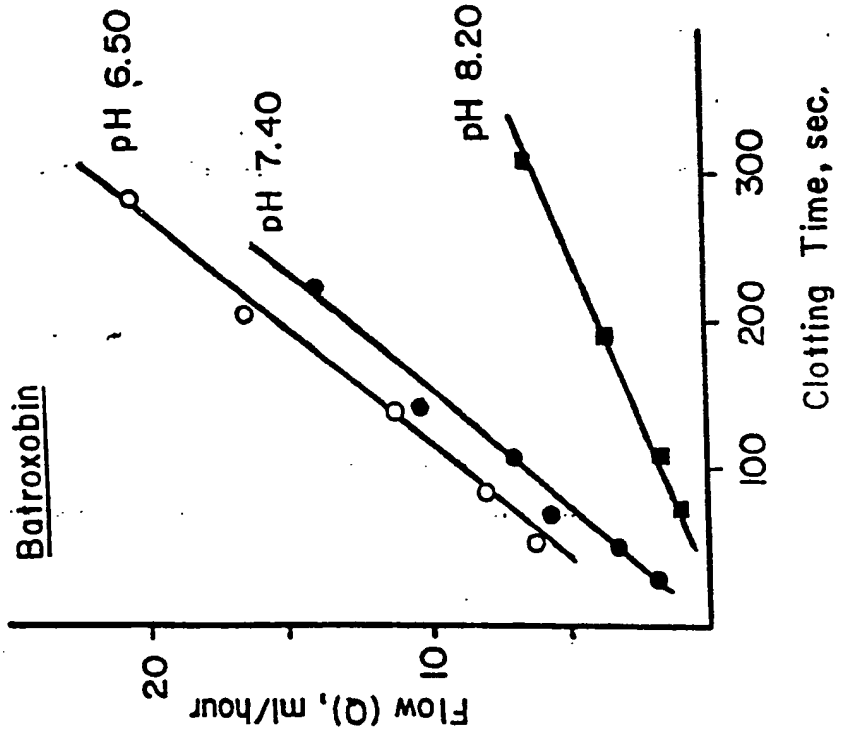

Batroxobin
pH 6.50
pH 7.40
pH 8.20
Flow (Q), ml/hour
20
10
100
200
300
Clotting Time, sec.

FIG. 2a.

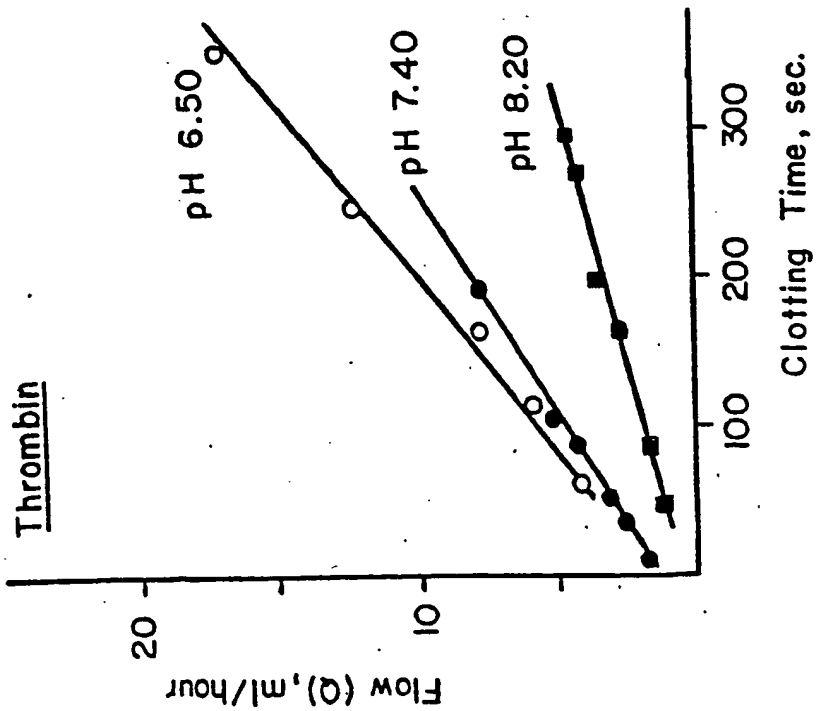
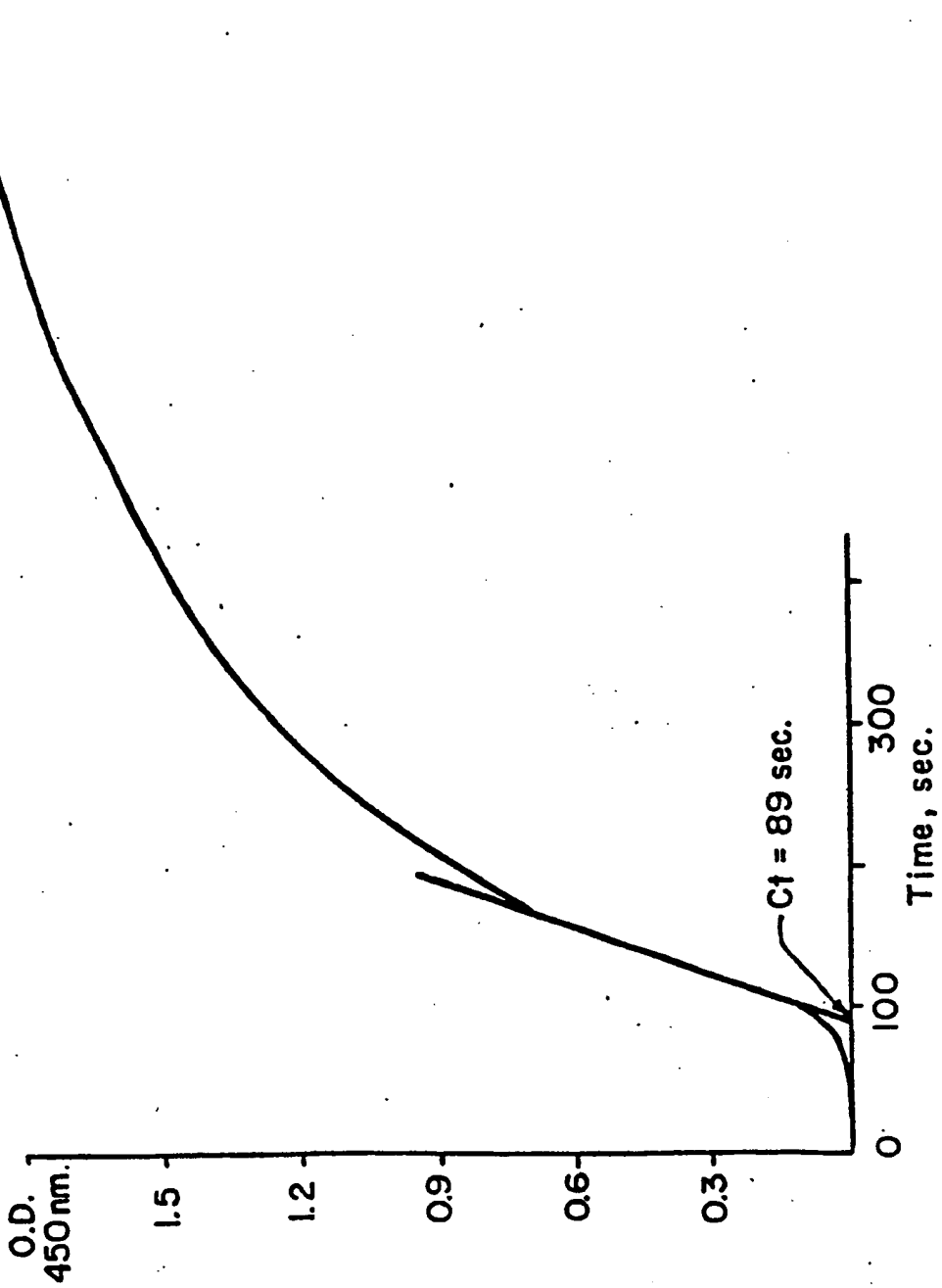

Thrombin
pH 6.50
pH 7.40
pH 8.20
Flow (Q), ml/hour
20
10
100
200
300
Clotting Time, sec.

FIG. 3.



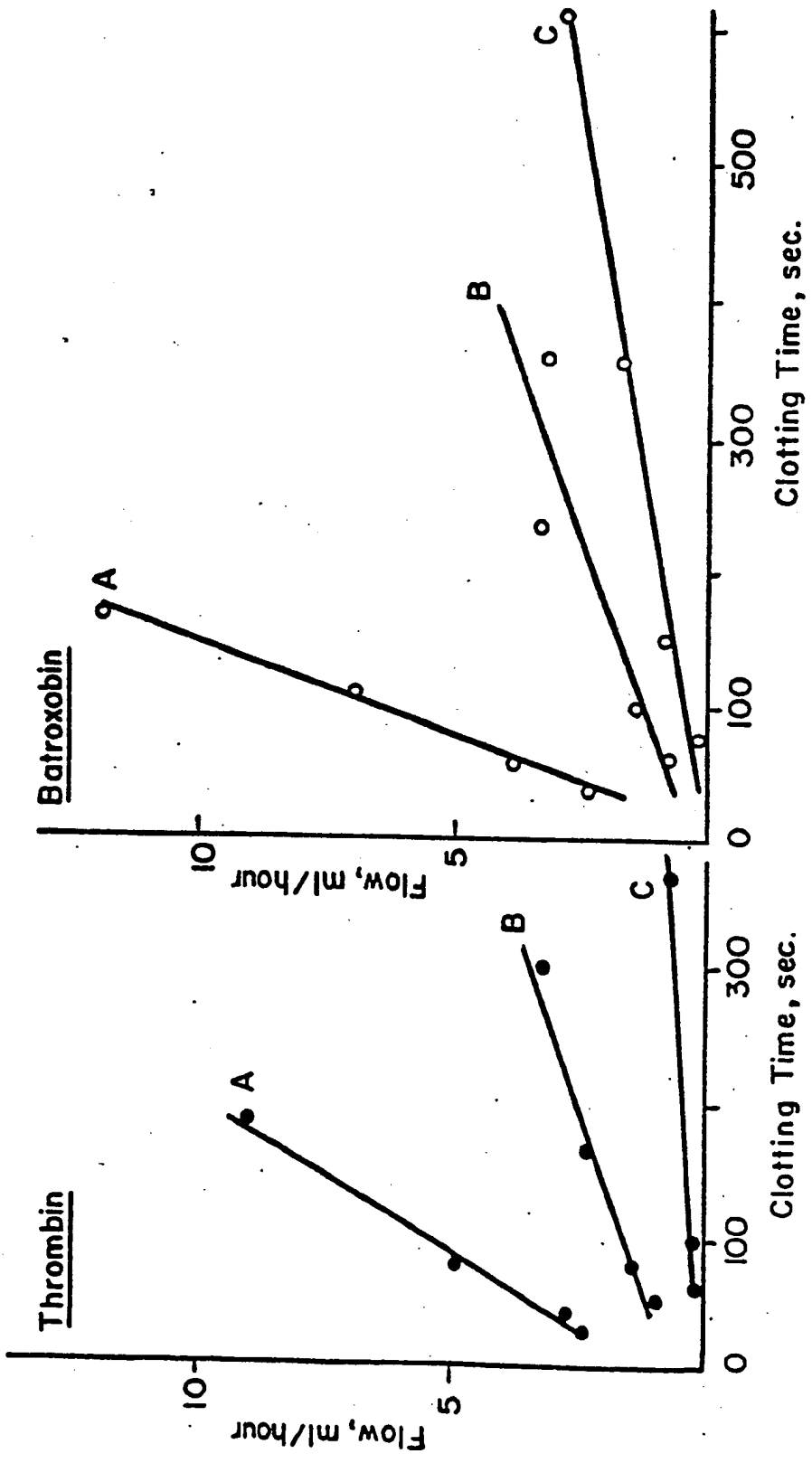

Batroxobin
A
B
C
10
5
0
Flow, ml/hour
0
100
300
500
Clotting Time, sec.

FIG. 4b.


Thrombin
A
B
C
10
5
0
Flow, ml/hour
0
100
300
Clotting Time, sec.

FIG. 4a.

FIG. 5b.

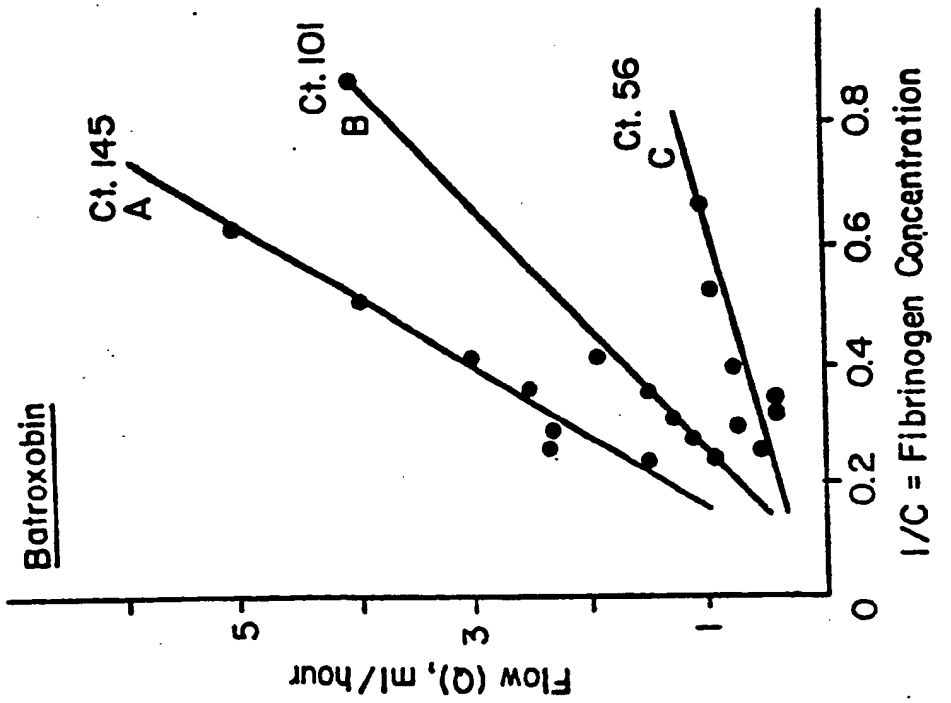

Batroxobin
Ct. 145
A
Ct. 101
B
Ct. 56
C
Flow (Q), ml/hour
5
3
1
0
0.2
0.4
0.6
0.8
1/C = Fibrinogen Concentration

FIG. 5a.

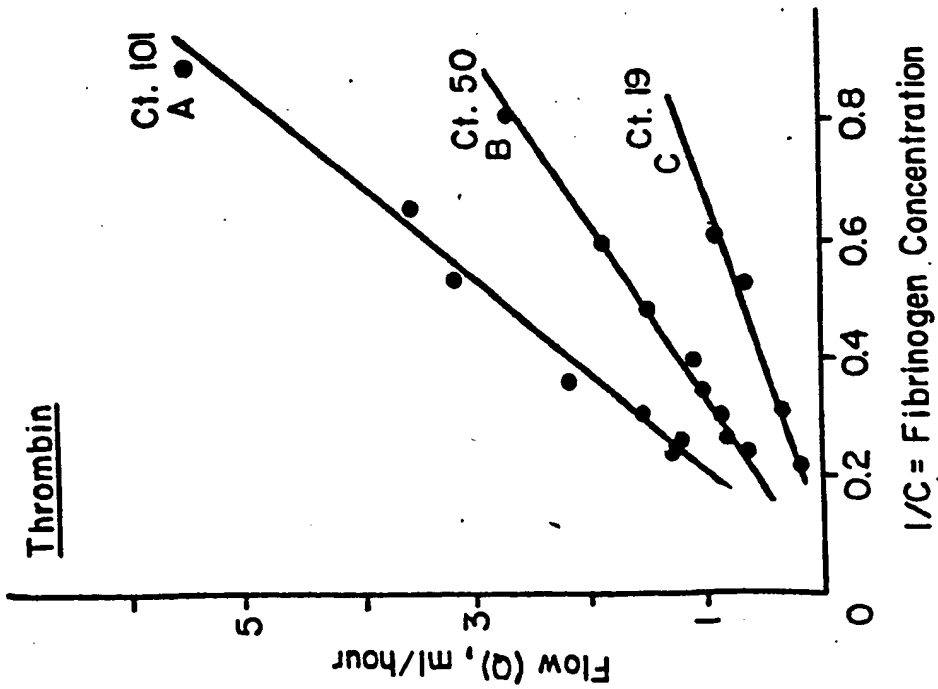

Thrombin
Ct. 101
A
Ct. 50
B
Ct. 19
C
Flow (Q), ml/hour
5
3
1
0
0.2
0.4
0.6
0.8
1/C = Fibrinogen Concentration

FIG. 6a.

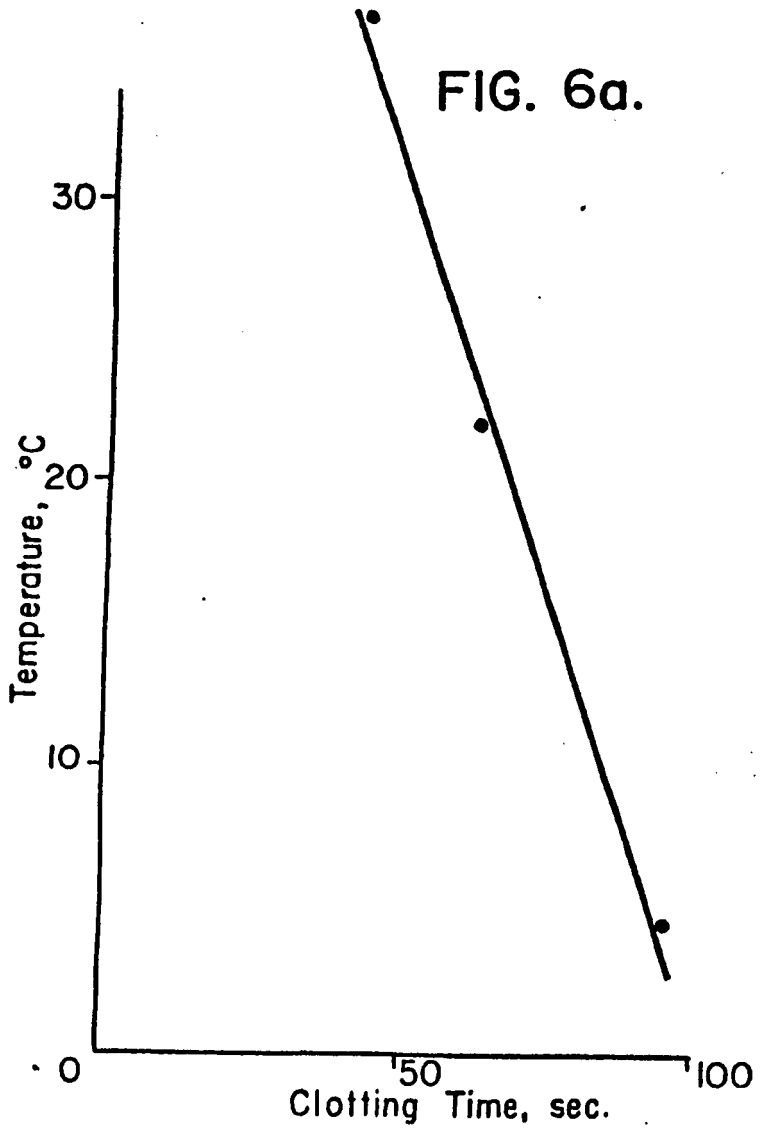



FIG. 6b.

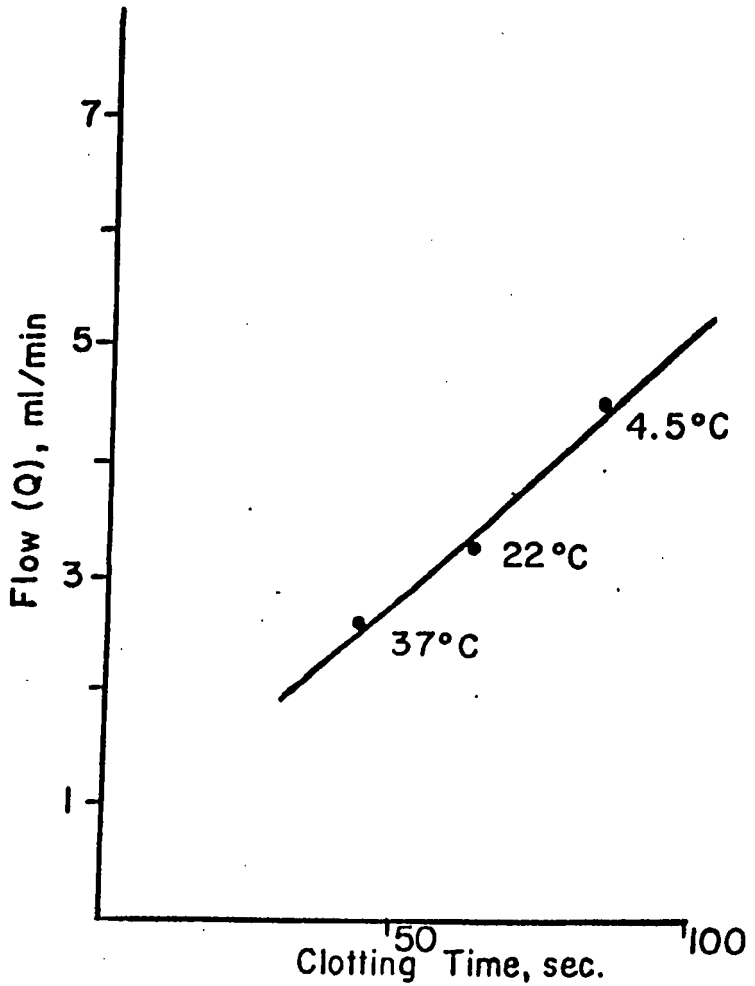





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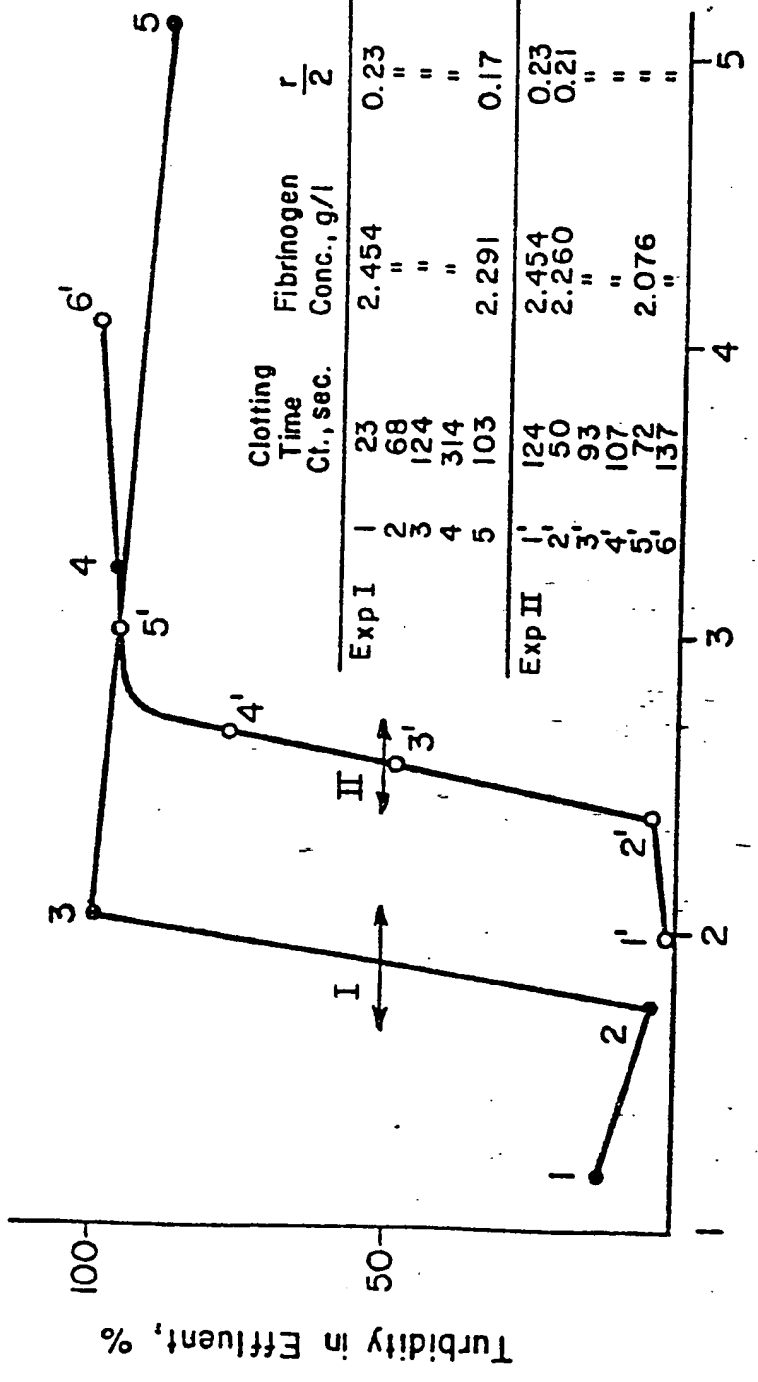
FIG. 7.




| | | Clotting Time Ct., sec. | Fibrinogen Conc., g/l | $\frac{r}{2}$ | Pore Diameter, μm |
|---|---|---|---|---|---|
| Exp I | 1 | 23 | 2.454 | 0.23 | 1.18 |
| | 2 | 68 | " | " | 1.74 |
| | 3 | 124 | " | " | 1.98 |
| | 4 | 314 | " | " | 3.17 |
| | 5 | 103 | 2.291 | 0.17 | 4.99 |
| Exp II | 1' | 124 | 2.454 | 0.23 | 1.98 |
| | 2' | 50 | 2.260 | 0.21 | 2.40 |
| | 3' | 93 | " | " | 2.55 |
| | 4' | 107 | " | " | 2.64 |
| | 5' | 72 | 2.076 | " | 2.99 |
| | 6' | 137 | " | " | 4.00 |

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# EUROPEAN SEARCH REPORT

0187894

Application number

EP 85 11 1133

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.4) |
|---|---|---|---|
| A | DE-B-2 407 961 (W.R. VIETH et al.)<br>* claims 1, 3, 6, 10; column 6, line 22 - column 7, line 30; examples 1-4 * | 1-4,12,14-17 | B 01 J 35/00<br>B 01 J 31/00<br>C 12 N 11/04<br>B 01 D 13/04 |
| D,A | CHEMICAL ABSTRACTS, vol. 84, no. 9, 1st March 1976, page 227, column 1, abstract no. 55888k, Columbus, Ohio, US; Y. INADA et al.: "Fibrin membrane endowed with biological function. II. Chloroplast adenosinetriphosphatase embedded in fibrin membrane", & BIOCHEM. BIOPHYS. RES. COMMUN. 1975 67(4), 1275-1280 | 1,12,14,15 | |
| D,A | CHEMICAL ABSTRACTS, vol. 88, no. 17, 24th April 1978, page 328, column 1, abstract no. 126372h, Columbus, Ohio, US; 27-12-1977; & - A - 77 156 912 (J. SHIMITZU) | 1,12,14,15 | |

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TECHNICAL FIELDS SEARCHED (Int. Cl.4)

B 01 D 13/04
B 01 D 39/14
B 01 D 39/16
B 01 J 31/00
B 01 J 35/00
C 12 N 11/02
C 12 N 11/04
C 12 P 21/00
G 01 N 33/50

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| BERLIN | 17-02-1986 | KUEHN P |

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L : document cited for other reasons

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| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.4) |
|---|---|---|---|
| A | CHEMICAL ABSTRACTS, vol. 91, no. 19, 5th November 1979, page 274, column 2, abstract no. 153576w, Columbus, Ohio, US; H. OKAMOTO et al.: "Fibrin membrane endowed with biological function. IV. Formation of cross-links between fibrinogen (or fibrin) and ribonuclease by transglutaminase", & ENZYME 1979, 24(4), 273-276 | 1,12, 14,15 | |
| A | CHEMICAL ABSTRACTS, vol. 93, no. 25, 22nd December 1980, page 376, column 1, abstract no. 234011b, Columbus, Ohio, US; Y. INADA et al.: "Fibrin membrane endowed with biological function. VI. Immobilization of multienzymes of the urea cycle", & BIOCHEM. BIOPHYS. RES. COMMUN. 1980, 96(4), 1586-1591 | 1,12, 14,15 | TECHNICAL FIELDS SEARCHED (Int. Cl.4) |
| A | CHEMICAL ABSTRACTS, vol. 92, no. 7, 18th February 1980, page 286, column 1, abstract no. 54218v, Columbus, Ohio, US; H. OKAMATO et al.: "Fibrin membrane endowed with biological function. V. Multienzyme complex of uricase, catalase, allantoinase and allantoicase", & BIOCHEM. BIOPHYS. ACTA 1980, 611(1), 35-39 | 1,12, 14,15 | |

-/-

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| BERLIN | 17-02-1986 | KUEHN P |

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0187894
Application number

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## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.4) |
|---|---|---|---|
| A | CHEMICAL ABSTRACTS, vol. 95, no. 26, 28th December 1981, page 379, column 1, abstract no. 225655d, Columbus, Ohio, US; & JP - A - 81 115 725 (GREEN CROSS CORP.) 11-09-1981 | 1,12, 14,15 | |
| A | EP-A-0 033 397 (J. KLEIN et al.) * claim 1 * | 1,12, 14-16 | |
| A | US-A-4 261 828 (G. BRUNNER et al.) * claims 1, 3 * | 1,12, 14 | |
| A | DE-A-2 009 515 (M. PETROW) * claim * | 2,3,4 | |
| | | | TECHNICAL FIELDS SEARCHED (Int. Cl.4) |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
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